Bacteriophages of *Erwinia amylovora* and their potential use in biological control

by

Jason J. Gill, B.Sc.

To sup?

A Thesis

submitted to the Department of Biological Sciences

in partial fulfillment of the requirements

for the degree of

Master of Science

April, 2000

Brock University

St Catharines, Ontario

Abstract

Forty-four bacteriophage isolates of *Erwinia amylovora,* the causal agent of fire blight, were collected from sites in and around the Niagara Region of Southern Ontario in the summer of 1998. Phages were isolated only from sites where fire blight was present. Thirty-seven of these phages were isolated from the soil surrounding infected trees, with the remainder isolated from aerial plant tissue samples. A mixture of six *E. amylovora* bacterial host strains was used to enrich field samples in order to avoid the selection bias of a single-host system. Molecular characterization of the phages with a combination of PCR and restriction endonuclease digestions showed that six distinct phage types were isolated. Ten phage isolates related to the previously characterized *E. amylovora* phage PEa1 were isolated, with some divergence of molecular markers between phages isolated from different sites. The host ranges of the phages revealed that certain types were unable to efficiently lyse some *E. amylovora* strains, and that some types were able to lyse the epiphytic bacterium *Pantoea agglomerans*. Biological control of *E. amylovora* by the bacteriophages was assessed in a bioassay using discs of immature pear fruit. Twenty-three phage isolates were able to significantly suppress the incidence of bacterial exudate on the pear disc surface. Quantification of the bacterial population remaining on the disc surface indicated that population reductions of up to 97% were obtainable by phage treatment, but that elimination of bacteria from the surface was not possible with this model system.

Acknowledgments

The author wishes to acknowledge the assistance of the following people:

My parents, for everything.

Dr. Antonet Svircev, for providing the author with invaluable advice, encouragement and material support, without which this work would not have been possible.

Dr. Alan Castle, who always managed to make time for consultations, for his indeterminate loans of research materials and for his input on the construction of the manuscript.

Dr. Yousef Haj-Ahmad and Dr. Peter Rand, for their advice and input on the direction of the study.

Alison Myers, who brought competent technical assistance and a sunny disposition to the project.

Ron Smith, for his provision of the materials and expertise required for the production of the transmission electron micrographs.

Brenda Beck, Charlene Green and Regina Grishkewish, for their technical assistance.

Dr. Al Jones, who generously provided the PCR primers and a bacterial strain which became integral parts of this project.

Art Moyer, for his donation of the immature pear fruit used in the bioassays.

Bill Lay, who provided an ample supply of pear blossoms for the bioassays.

Dr. Karen Bedford, for her donation of bacterial strains.

Dr. David Hunter, for his advice on all things pomological.

Neva Grieg, Leo Van Driel and Kathy Whybourne, for their assistance, advice and timely loans of equipment.

This project was funded in part by grants from the Ontario Apple Marketing Commission and Science Horizons.

Table of contents **Page**

List of tables **Page**

List of figures **Page**

1 Introduction

1.1 Historical background of fire blight

In 1880, fire blight became the first disease of plants to have a bacterium identified as its causal organism (Burrill, 1880). Initially named *Micrococcus amylovorus* by Burrill, the pathogen was later called *Bacillus amylovorus* and finally *Erwinia amylovora* (Burr) Winslow et al., the species name which it holds today.

E. amylovora is believed to be indigenous to North America, as all of the earliest reports of infections (from 1780 to the early 1900's) come from this continent (Stewart, 1913; Thomson, 1992). After 1900, fire blight was reported in New Zealand (1919), Mexico (1943), England (1957) and Egypt (1964). Since its introduction in England and Egypt, the pathogen has spread through most of Europe and the Middle East (van der Zwet, 1996).

The economic impact of the disease is difficult to predict due to its sporadic nature. Apple or pear orchards may endure several years untouched by fire blight, only to be completely destroyed in one or two seasons (Jackson, 1910; Stewart, 1913). A prime example of the potential of *E. amylovora* to eradicate entire orchards is cited in van der Zwet and Keil (1979), where an outbreak from 1902-1904 killed 99% of all pear trees growing in two California counties.

1.2 The host plant of *E. amylovora*

While *E. amylovora* is capable of colonizing and damaging most species of the family Rosaceae, including plum (*Prunus*), quince (*Cydonia*), raspberry (*Rubus*), *Cotoneaster* and hawthorn (*Crataegus*), it is primarily of concern in apple (*Malus X domestica* Borkh.) and pear (*Pyrus communis* L.) (Stewart, 1913; van der Zwet and

Keil, 1979).

Apple and pear trees are typically not grown as whole trees. Bud-bearing wood cut from apple or pear trees (the scion) is grafted onto a compatible tree (the rootstock), which may be propagated either from seed or clonally from cuttings. Scion cultivar and rootstock combinations are usually selected by individual growers based on recommendations of extension personnel, cost, past experience and perceived market demands for fruit varieties.

A large number of apple and pear scion cultivars are available to growers. Among other qualities, differences in fruit size, colour and flavour, tree vigor and disease resistance are exhibited by various cultivars. Popular apple varieties in Ontario orchards such as Red Delicious and McIntosh are rated as possessing moderate to high resistance to fire blight, while Gala and Ida Red, among others, are highly susceptible (Thomas and Jones, 1992; van der Zwet and Beer, 1995). In pear, some of the most popular commercial cultivars such as Bartlett, Bosc and Flemish Beauty are highly susceptible (van der Zwet and Beer, 1995).

Different rootstocks, while exhibiting disease resistance phenotypes of their own, are also able to impart this trait to their scion. Clonal apple rootstocks such as M.26 and M.9, common in Ontario and used for their dwarfing ability, increase the susceptibility to fire blight of the scion (van der Zwet and Keil, 1979; van der Zwet and Beer, 1995; Travis et al., 1998).

1.3 Disease symptoms of fire blight

Fire blight symptoms can appear on any part of the tree, from blossoms to roots. The name of the disease signifies both the appearance of infected tissue and the speed with which the bacterium is able to destroy entire plants and orchards. Symptoms

typically appear first in blossoms or new shoot growth as a water-soaked appearance followed by necrosis (Figure 1A). Necrotic blossoms may remain attached to the tree even into the winter months. Symptomatic leaves turn dark brown in apple or black in pear, with the tips of infected shoots often taking on a characteristic "shepherd's hook" appearance (Figure 1B).

Necrosis is often accompanied by the production of bacterial exudate commonly referred to simply as "ooze" (Figure 1C), which is composed primarily of bacterial cells and serves as a major inoculum source for infections of neighboring plants. While succulent tissue is most susceptible to *E. amylovora*, the pathogen can migrate into the woody tissue and cause the formation of cankers (Figure 1D). Especially susceptible is the graft union between the rootstock and scion, at which point necrotic tissue may girdle the base of the tree preventing nutrient transport.

1.4 Life cycle of *E. amylovora*

1.4.1 Primary infection

E. amylovora begins its annual infection cycle in early spring with activation of the bacterial population residing in overwintering cankers (Figure 2). Cankers are necrotic regions established in the woody tissues of the tree. The actively growing bacterial cells located in the margins of some cankers are extruded onto the bark surface and may be visible as white to red droplets if present in sufficient quantity. Larger cankers with indeterminate (smooth) borders were found by Beer and Norelli (1977) to have a higher probability of producing viable cells on the bark surface.

The bacteria exposed on the canker surface may be disseminated by a number of external vectors to other areas of the same tree or other trees in the vicinity. Insects have been known to be vectors of the disease for many years (Stewart, 1913; Orton

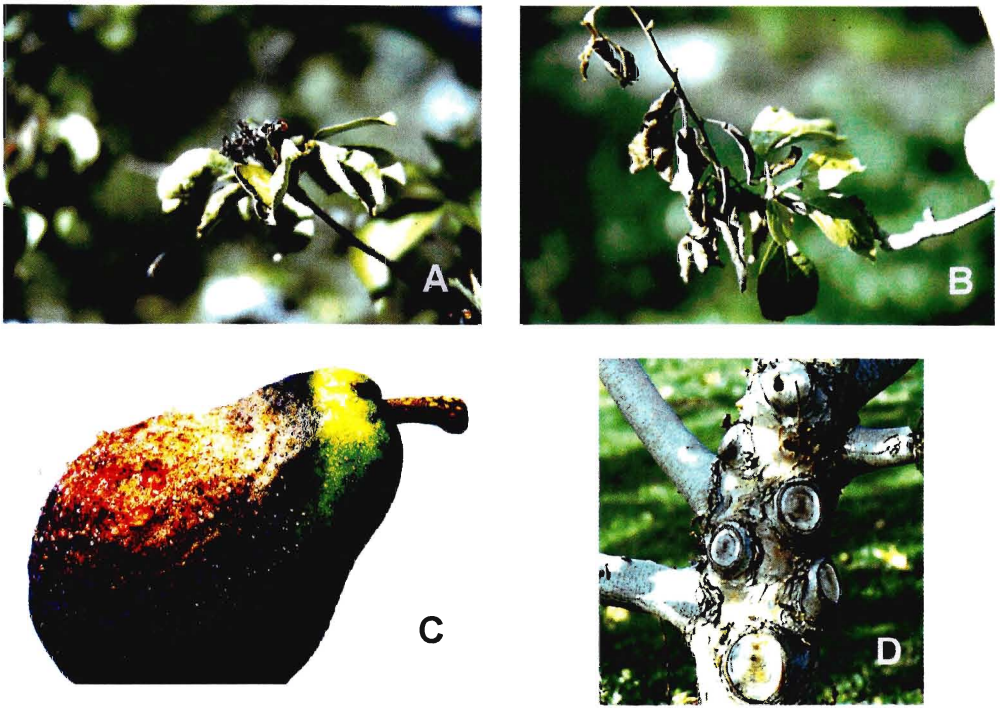

Figure 1. An illustration of the symptoms of fire blight in various tissues. **A:** blighted apple blossoms. **B:** An apple shoot showing fire blight symptoms; note the characteristic "shepherd's hook" shape of the shoot tip. **C:** A pear fruit exhibiting advanced fire blight symptoms; note the droplets of bacterial exudate, or ooze, covering the fruit surface (from van der Zwet and Beer, 1995). **D:** Attempts to prune out cankers on this Ida Red apple tree have failed, as cankers have become established on the trunk.

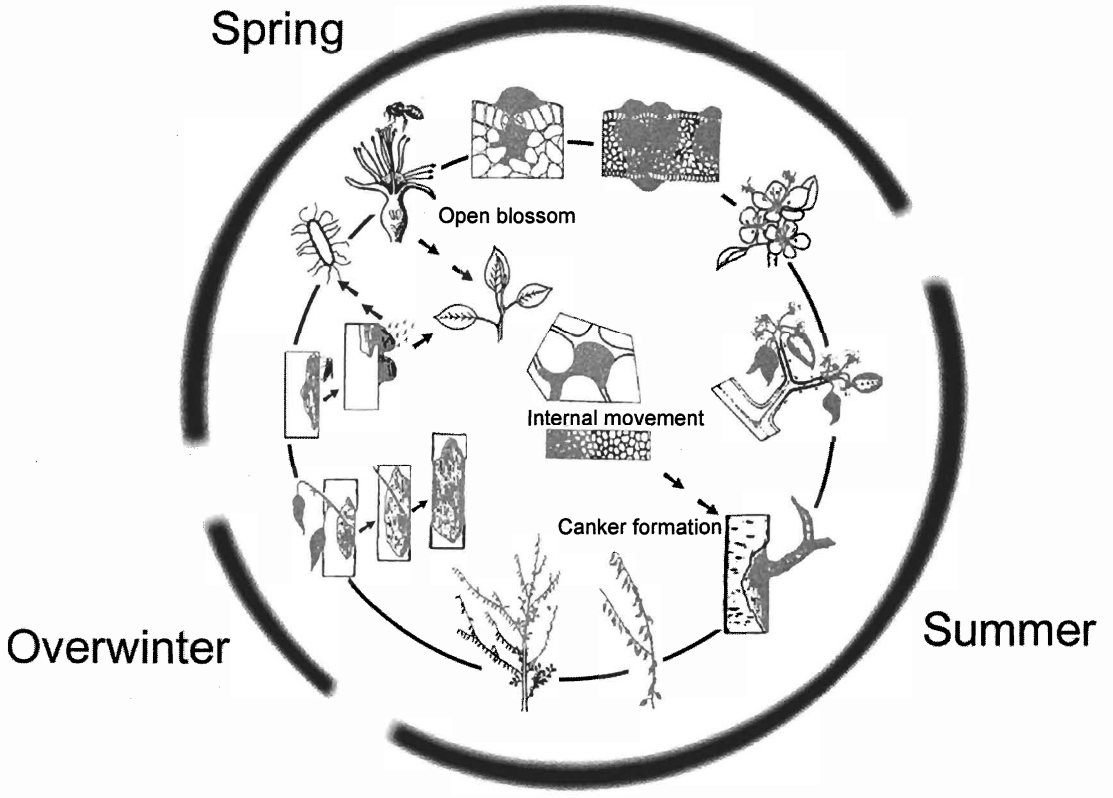

Figure 2. The life cycle of *E. amylovora,* causal agent of fire blight in apples and pears. The cycle begins in early spring, with the release of bacterial exudate from overwintering cankers. The pathogen is then transported to open blossoms or young tissue, from which it can spread into the woody tissues of the tree to form cankers. Figure adapted from van der Zwet and Beer, 1995.

and Adams, 1915; Hildebrand, 1937). Flies (Miller and Schroth, 1972; Thomson et al., 1999), among other insects, have been implicated in transmission of the disease. Honey bees do not generally visit active cankers but they are capable of transmitting the pathogen from diseased blossoms to healthy ones (Thomson et al., 1992).

It has been long established that the primary site through which the pathogen gains entry to the tree are opened blossoms (Jackson, 1910; Stewart, 1913; Thomas and Ark, 1934; Hildebrand, 1937). *E. amylovora* is initially deposited on the stigmas of open blossoms, where cells undergo a period of epiphytic growth (Thomson, 1986). This period is crucial for the subsequent development of infection, as the bacterial population can greatly increase in size while in this epiphytic stage reaching a total population of 10^6 to 10^7 CFU/stigma (Johnson and Stockwell, 1998). Scanning electron microscopy of inoculated apple blossoms indicates that the bacteria are localized to the stigma of the blossom, with no cells visible on the anthers, style or nectaries (Hattingh et al., 1986). Apple and pear stigmas bear columnar papillae and a sticky exudate which can contain sugar concentrations as high as 60% (Thomas and Ark, 1934). While *E. amylovora* has been demonstrated to survive under high osmotic stress (Pusey, 1999), the presence of moisture in the form of rain or dew plays an important role in the progress of the disease as it dilutes sugars on the hypanthium to osmotic potentials which are more suitable for bacterial growth. It also redistributes this epiphytic *E. amylovora* population from the stigma of the flower to the hypanthium, where it gains entry to the interior of the plant through secretory pores called nectarthodes (Hildebrand, 1937; Thomson, 1986; Pusey, 1997). *E. amylovora* moves through the intracellular spaces causing rapid necrosis in infected blossoms, and may migrate down the peduncle into the body of the tree to establish a canker in woody tissue.

In crops of pear and some apple varieties, secondary blossoms ("rattail blossoms") may continue to appear long after primary bloom. In areas where cooler spring weather is the norm, such as Ontario, sufficient inoculum to cause severe outbreaks of the disease in blossoms may not be available until after primary bloom (Covey and Fischer, 1988). In these cases, most blossom blight occurs in secondary blossoms (Dueck and Quamme, 1973; Covey and Fischer, 1988).

1.4.2 Secondary infection

Primary infections increase the amount of inoculum available in an orchard to cause secondary infections, either through wounds or secondary blossoms. Insects with chewing or sucking mouthparts such as aphids are able to transmit the pathogen from infected trees to healthy tissues (Stewart, 1913). Airborne water is a major vector for transmission of the fire blight pathogen. Wind speeds typically found in an orchard environment during the growing season are sufficient for dispersal of water droplets over a distance at least 1 m, the distance between typical orchard rows (Bauske, 1971). Violent weather such as rain or hail storms can be especially effective in the distribution of the pathogen from established primary infections, as aerosols containing the pathogen can be produced (McManus and Jones, 1994). Such weather also generates potential infection sites in the form of small breaks in the tree bark or in the cuticle of new shoot growth through which the pathogen may enter (Crosse et al., 1972).

Once established in the intracellular space, the pathogen is able to travel within the plant, causing tissue necrosis in young tissue which may also lead to canker formation. Such late season infections may be more damaging to the tree than primary infections, as the pathogen appears to travel through the tree to the rootstock more rapidly later in the season (Momol et al., 1998).

The bacterial population of a canker may be distributed within the tree via the vascular system and cause symptom development in the current season's succulent growth (Hickey et al., 1999) or in the rootstock (Momol et al., 1999). The presence of the pathogen inside healthy tissue does not always correlate with development of disease symptoms. It is not currently known why apparently asymptomatic tissue is able to harbor a pathogen population (Ge and van der Zwet, 1995; Momol et al., 1998).

1.5 *Erwninia amylovora*

Erwinia amylovora, the causal agent of fire blight, is a Gram negative rod, motile by peritricious flagella. In pathogenesis, *E. amylovora* cells do not enter the host plant cells, but remain in the intercellular space, or apoplast (Walters et al., 1990; Alfano and Collmer, 1996). Despite the fact that *E. amylovora* was the first bacterium ever determined to cause disease in plants, major advances in unraveling the mechanism of its pathogenicity were not made until this decade.

The genes which are responsible for pathogenicity have been designated *hrp* (for *h*ypersensitive *r*eaction and *p*athogenicity) genes; these genes are also responsible for triggering a hypersensitive response in non-host plants. The *hrp* genes of most species are organized into a 23-25 kilobase pair cluster of 6-8 operons under the control of a common promoter protein forming a regulon, and this cluster is sometimes referred to as a "pathogenicity island" or "*hrp* island" (He, 1998). The *hrp* genes of several bacterial plant pathogens show high sequence similarity to each other and to the virulence components of animal pathogens such as *Yersinia*, *Salmonella* and *Shigella*, and these genes code for components of a type III secretion system (Kim et al., 1997; Gaudriault et al., 1998; He, 1998).

Also playing a role in the pathogenicity of *E. amylovora* is its production of a

copious capsule composed of exopolysaccharide (EPS) (Bennett and Billing, 1978). It has been shown that *E. amylovora* strains lacking the ability to produce EPS lose most or all of their virulence *in vivo* (Bennett and Billing, 1978). The exact role of EPS in pathogenicity is not fully understood, but it is believed to protect the cell from recognition by the host plant. In studying the effects of high sugar concentrations on the growth of *E. amylovora*, Pusey (1999) noted that an acapsular strain of the pathogen exhibited reduced survival in a high sugar environment similar to what exists on the blossom surface, indicating that EPS may also contribute to osmotolerance. The amount of EPS required to allow pathogenicity is apparently quite small as *E. amylovora* constitutively expressing the capsular depolymerase enzyme of bacteriophage PEa1(h) is still pathogenic, although virulence in immature pear fruit is reduced (Hartung et al., 1988).

1.6 Control of fire blight

1.6.1 Orchard management

Early removal of infected plant tissue from the orchard is crucial in preventing further spread of *E. amylovora* within trees and to keep the overall level of potential inoculum low. Established cankers can be excised from woody tissue. Due to the rapid migration of *E. amylovora* through the tree, cuts into woody tissue are made 20-30 cm from the canker margin; care must be taken to sterilize tools between cuts to prevent reinfection (Travis and Kleiner, 1997, Thomson, 1992). It was shown by van der Zwet and Keil (1979) that serious outbreaks of the disease could be controlled by pruning alone, but this reduced the trees to one third of their original size. Extensive pruning, however, has been shown to be more economically sound than allowing infected material to remain attached to the tree, a practice which ultimately results in the loss of

the whole tree (Norelli and DeMarree, 1999). Frequent patrols of the orchard in order to identify and excise fire blight infections are effective but may be impractical on the scale of a modern commercial orchard.

As *E. amylovora* is indigenous to North America, it is presumed that it evolved on plants such as hawthorn, crab apple and mountain ash (*Sorbus*), Rosaceous genera which are also indigenous to this continent (Stewart, 1913; Thomson, 1992). Stands of such potential host plants located in the vicinity of apple or pear orchards can serve as reservoirs of fire blight inoculum. In English orchards, hawthorn hedges are commonly found bordering apple or pear orchards and have been implicated in numerous outbreaks of fire blight. Berrie and Billing (1996) reported that disease symptoms appeared in pear trees more than 100 m from blight-infested hawthorn hedges, the presumed source of primary inoculum. Prevention of the disease in commercial orchards must therefore include its prevention in local populations of potential alternate hosts.

1.6.2 Chemical control

Historically, the use of broadly toxic compounds containing copper, typically Bordeaux mixture (a mixture of copper sulphate and lime), copper ammonium carbonate or copper hydroxide have been used for the control of fire blight (Ark, 1953; van der Zwet and Keil, 1979). Copper treatments give some control of the disease but are broadly phytotoxic, associated with leaf spotting and fruit russeting (Ark, 1953; Steiner, 1998). This potential of copper to lower fruit quality has made it an unattractive option for growers. In the early 1950's, studies indicating that antibiotic compounds could control fire blight without phytotoxic side effects were released (Ark, 1953; Heuberger and Poulos, 1953). Streptomycin became the bactericide of choice to

control *E. amylovora* in North America (Thomson, 1992).

Strains of *E. amylovora* which are resistant to streptomycin have arisen in many of North America's major apple and pear producing regions (Miller and Schroth, 1972; Moller et al., 1972; Schroth et al., 1978; Loper et al., 1991). Resistance was first reported in California pear orchards in the early 1970's (Miller and Schroth, 1972; Moller et al., 1972). Strains of the pathogen resistant to streptomycin are believed to arise as a series of separate events and spread locally, rather than descending from a common ancestor (McManus and Jones, 1994a). Alleles which confer streptomycin resistance have been located on the chromosome (Schroth et al., 1978) and on various episomal elements (Chiou and Jones, 1991; McManus and Jones, 1994a). In the study by Chiou and Jones (1991), resistance genes contained in plasmids were found to bear sequence similarity to those found in *P. syringae* papulans; this finding was coupled with a high frequency of transfer of this element (up to 1 in 4.5 $x10^2$). Researchers in the field have noted that strains of *E. amylovora* sensitive to streptomycin have now become the exception rather than the rule in Washington and Oregon orchards (V. Stockwell, personal communication).

In Ontario, adequate control of the disease is still obtained with a combination of orchard management and streptomycin. An agricultural formulation of this antibiotic, Streptomycin 17, is currently the only chemical treatment officially recommended for the control of fire blight (OMAFRA, 1996).

1.6.3 Predictive models

Fire blight outbreaks coincide with periods of warm temperatures followed by rain or heavy dew, a trend first noted in 1817 by William Coxe (Stewart, 1913). Warm temperatures present before and during bloom have been implicated in economically

severe fire blight epiphytotics (Reil et al., 1979). The use of predictive models in determining the optimal times for application of antibiotics have enhanced their effectiveness while reducing the number of applications required to obtain adequate control. Timing is especially important when one considers that the principal antibiotic, streptomycin, is non-systemic and is effective on the plant surface for only 48 to 72 hours after application (Thomson, 1992).

An early predictive model proposed by Thomson et al. (1982) recommended antibiotic application only when the daytime temperature exceeded a line drawn on a time versus temperature graph from 16.7 °C on March 1 to 14.4 °C on May 1. While this model does not take into account other environmental conditions such as precipitation, it was able to predict with reasonable accuracy the development of disease symptoms.

A model proposed by Zoller et al. (1979) predicted fire blight outbreaks by observing the cumulative degree hours (CDH) above a temperature of 18.3 °C. The software-based model MARYBLYT (Steiner and Lightner, 1992) also uses this criterion, in conjunction with precipitation patterns and orchard status to predict periods of epiphytic bacterial growth. It then recommends bactericide application immediately prior to rain or wet weather which triggers an infection event. The MARYBLYT program predicts infection events in blossoms based on the accumulation of sufficient degree hours (110 CDH) above 18.3 °C to allow epiphytic growth, followed by a wetting period which transfers the epiphytic population to susceptible sites in the open blossom. The model begins at green tip and continues through the end of primary bloom; it is less able to predict infections in secondary blossoms (van der Zwet et al., 1994)

Evaluations of this model have indicated that MARYBLYT is able to predict outbreaks of fire blight in blossoms with reasonable accuracy, and reduced the number

of antibiotic applications required to obtain control of the disease at primary bloom (Jones, 1992; van der Zwet et al., 1994). An assumption made by MARYBLYT is that inoculum levels in an orchard are always high; newer models, such as COUGARBLYT, also take into account the past incidence of fire blight strikes within the orchard and in the surrounding area. This is expected to reduce the amount of unnecessary antibiotic applications as areas with no history of fire blight are less prone to harboring large pathogen populations.

1.6.4 Biological control

1.6.4.1 Principles of biological control

Biological control utilizes the abilities of benign organisms to control populations of undesirable organisms. Control can be achieved through the production of a compound which is inhibitory to the target organism and/or by competition with the target organism for required nutrients or protected growth sites. An effective biological control agent exploits a weakness in the pathogen (Schisler and Slininger, 1997). *E. amylovora* requires a period of epiphytic growth on the blossom surface in order to reach sufficient numbers to initiate an infection, and the blossom is the primary point of entry of the pathogen into the tree. It is the blossom, therefore, which has been the focus of most biological control measures for fire blight (Pusey, 1997; Johnson and Stockwell, 1998; Pusey, 1999).

The effectiveness of a biological control agent depends greatly on its ability to successfully and independently colonize the niche of the pathogen and exert its control activity (Janisiewicz, 1999). Selection of a biological control agent should therefore include screening of candidates in a system which closely mimics the conditions under which they would be expected to perform in regular field use (Schisler and Slininger,

1997). Antagonists which control pathogen populations well in artificial media may be unable to survive in the niches for which the pathogen is adapted; in the case of biological control on the blossom surface, the ability to withstand conditions of high osmotic stress and dessication are essential in a biological control agent (Pusey, 1999).

The two most prevalent model systems for the evaluation of biological control potential against *E. amylovora* are the use of immature pear fruit (Vanneste et al., 1995; Vanneste et al., 1996) and detached blossoms (Pusey, 1997). Both of these systems have been shown to detect microbial strains antagonistic to *E. amylovora*.

1.6.4.2 Biological control of *E. amylovora*

Interactions between *E. amylovora* and other nonpathogenic epiphytic bacteria have been documented for some time. A study by Thomson et al. (1976) reported that saprophytic bacteria collected from the orchard produced control activity against fire blight infections similar to streptomycin or Kocide. A yellow saprophytic bacterium, probably an isolate of *Pantoea agglomerans* (formerly *Erwinia herbicola*) was also shown to prevent symptom development by *E. amylovora* when coinoculated onto immature pear slices (Erskine and Lopatecki, 1975). Isolates of *Bacillus subtilis* have also been reported to prevent fire blight (Zeller and Wolf, 1996). These findings, coupled with the documented rise in streptomycin resistance reported in the 1970's, made biological control agents against fire blight an attractive option.

The biological control agent *Pseudomonas fluorescens* A506 has been marketed as BlightBan A506 (Plant Heath Technologies, Boise, ID) since 1996 (Lindow, 1998) and has been shown to successfully control fire blight in apple and pear (Wilson and Lindow, 1993; Stockwell et al., 1998). A second biological control agent also marketed under the BlightBan name, *E. herbicola* C9-1 is currently awaiting approval.

While both of these agents are able to colonize sites in the open blossom and exclude *E. amylovora* (Wilson et al., 1992; Wilson and Lindow, 1993), the modes of action of these two biological control agents differ significantly. *P. fluorescens* A506 is able to colonize stigmatic surfaces and appears to compete with *E. amylovora* for nutrients and protected sites (Wilson and Lindow, 1993). A number of *E. herbicola* isolates have been shown to produce an antibiosis effect against *E. amylovora* (Wilson et al., 1992; Vanneste et al., 1992; Kearns and Hale, 1996).

Application of biological control agents must follow blossom opening but precede inoculation of *E. amyolovora* to the stigmatic surface by sufficient time to allow the establishment of the biological control agent population. Environmental conditions in the blossom may have an impact on the efficacy of biological control agents, as both *P. fluorescens* A506 and *E. herbicola* C9-1 are less osmotolerant than *E. amylovora* (Pusey, 1999). Such agents may therefore be of limited use in certain climates.

1.7 Bacteriophages

Bacteriophages are the largest group of viruses, utilizing species in the eubacteria and archaebacteria as hosts (Ackermann and Dubow, 1987). As viruses, they do not possess their own metabolism, but must rely on the cellular mechanisms of a host cell in order to complete their life cycle. Phages consist of a genome, which can be comprised of either single or double stranded DNA or RNA, which is enclosed within a proteinaceous coat.

A 1995 survey of the literature counted 4551 published morphological descriptions of bacteriophages (Ackermann, 1996). Of these, 96% percent are tailed, with only 4% being either cubic, filamentous or pleomorphic. Most of the documented non-tailed phages represent small virus families which utilize uncommon species as

hosts, such as mycoplasmas and archaebacteria (Ackermann, 1987; Ackermann, 1996). Tailed phages are of the order *Caudovirales*, which contains three families: *Myoviridae* (long, contractile tails), *Siphoviridae* (long, non-contractile tails) and *Podoviridae* (short, non-contractile tails) (Maniloff and Ackermann, 1998). These families correspond to the morphological groups A, B and C, respectively, initially described by Bradley (1965) and which have since been elaborated upon as shown in Figure 3. Bacteriophages of the order *Caudovirales*, and therefore the majority of known bacteriophages, contain a genome of linear, double-stranded DNA (Ackermann, 1987)

While phages have been placed into these families based on gross morphology of the virion and its type of nucleic acid, the organization of phages into genera and species is less clear. The concept of a species as comprised of members of a closed interbreeding population does not readily apply to asexual organisms such as bacteriophages (Campbell, 1988). The International Committee on the Taxonomy of Viruses (ICTV) has therefore defined a virus species as "a polythetic class of viruses that constitutes a replicating lineage and occupies a particular ecological niche" (Van Regenmortel et al., 1997). Placement of a given phage into a particular genus relies on resemblance to certain type species based on its possession of characteristics such as concatamer formation and nucleic acid cleavage during progeny phage assembly, and the presence of particular molecular characteristics such as virus-encoded polymerases or inclusion of unusual DNA bases into the phage genome (Maniloff and Ackermann, 1998).

The nomenclature of bacteriophages at the genus or species level does not currently adhere to the latin binomial system nor to any other universally recognized standard (Ackermann, 1987, Ackermann, personal communication). Phage names

such as T4, P1 and λ have been in common use for too long to be changed, and novel phages are often named with combinations of ϕ, letters and numbers which bear no inherent meaning to anyone outside of the discovering researcher's laboratory. One convention suggested by Ackermann et al. (1978) utilizes the first initials of the phages' host bacterial species followed by a unique number; this convention will be followed for the naming of phages isolated in this work.

1.7.1 Life cycle

Attachment of the bacteriophage to the host cell triggers the release of the viral genome and associated proteins into the cell, at which point the viral genome regulates the production of new virions (Figure 4). This lytic pathway results in the eventual lysis of the host cell with the release of the assembled virus particles. A secondary form of life cycle exhibited by many bacteriophages is lysogeny, in which the phage nucleic acid does not direct the formation of new virus particles, but instead incorporates itself in to the bacterial chromosome or exists as an episomal element. This dormant phage genome, or prophage, replicates with the bacterial cell until it is reactivated and resumes its lytic pathway. The mechanisms of reactivation vary between phages, but are usually triggered when the host cell is placed under stress, either from DNA damaging agents or environmental conditions.

1.7.2 Bacteriophages as biological control agents

The specificity of a bacteriophage for a given bacterial species or strain has been well documented (Adams, 1959; Dulbecco and Ginsberg, 1988), a property which allows targeted suppression of a particular bacterial species within a population. The

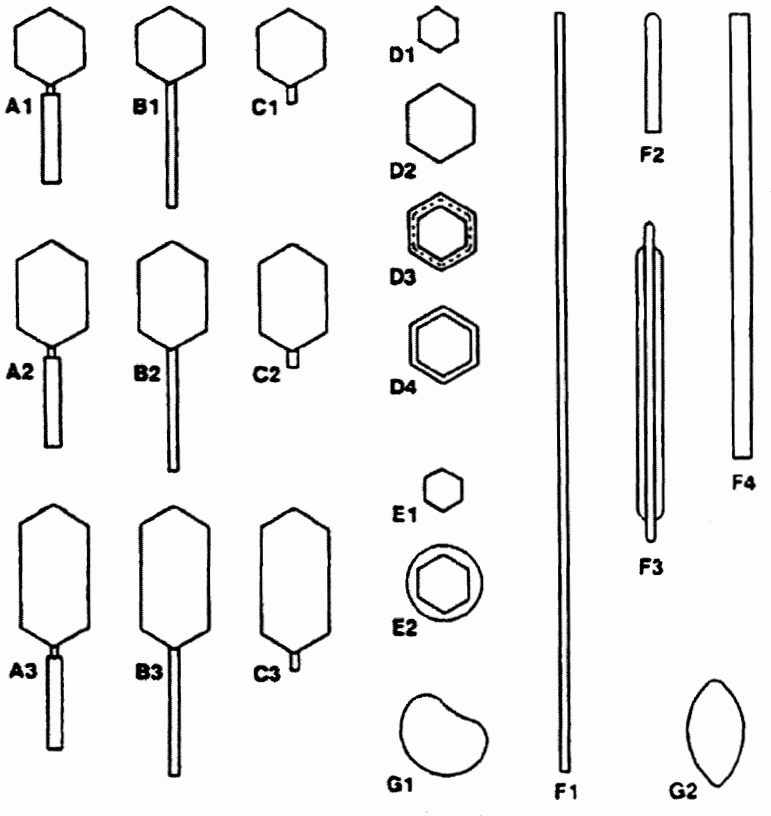

Figure 3. Morphotypes of bacteriophages. Types A-F were originally proposed by Bradley (1965). Types A, B and C possess an icosahedral head with a tail which is either long and contractile, long and non-contractile, or short and non-contractile, respectively. Types D and E possess no tail with either large or small capsomeres, respectively. Type F phages are filamentous, and type G phages are pleomorphic. Types have been subdivided based on capsid shape and fine structure
Figure taken from Ackermann (1996).

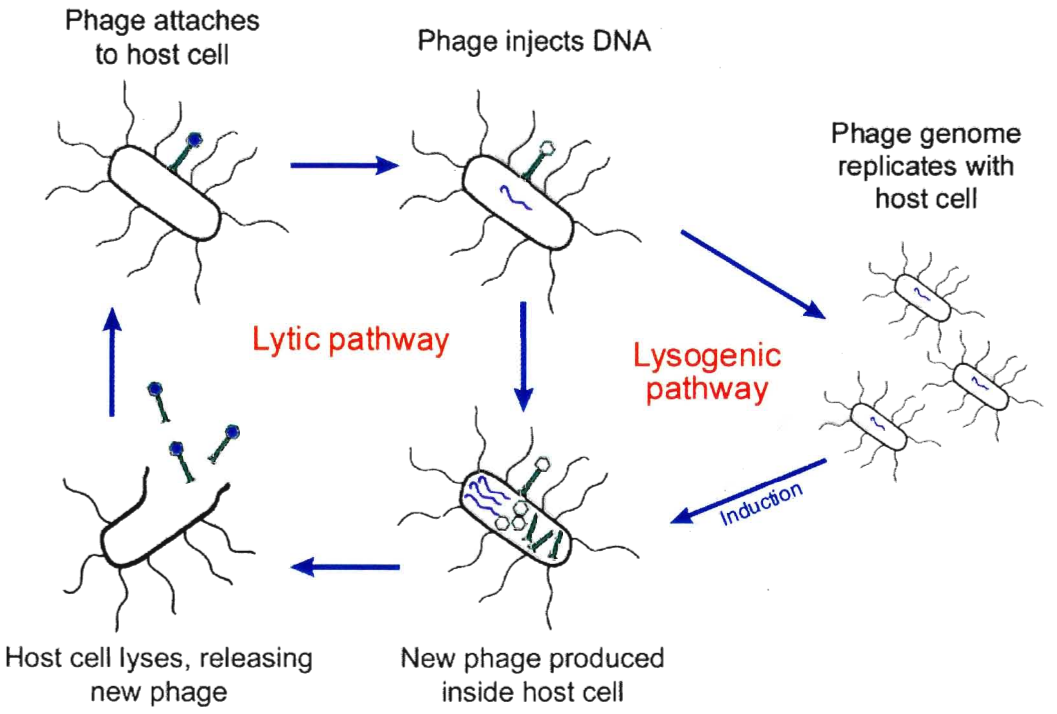

Figure 4. Life cycle depicting the lytic and lysogenic pathways of a typical bacteriophage.

use of bacteriophages as potential biocontrol agents has been historically criticized (Okabe and Goto, 1963, Vidaver, 1976), however, despite reports of phages effectively treating human diseases such as cholera (Ackermann and Dubow, 1987).

Several workers have had variable levels of success in controlling bacterial pathogens using bacteriophages (Gill et al., 1999; Schnabel et al., 1999; Palmer et al., 1997; Munsch et al., 1995; Jones et al., 1998; Civerolo, 1969). Munsch et al. (1995) reported that the use of a single bacteriophage isolate, TO 1, reduced bacterial blotch of mushrooms caused by *Pseudomonas tolaasii* by 30 to 80%, with no rise in a phage-resistant bacterial population over the course of 10 years.

Jones et al. (1998) demonstrated that mixtures of bacteriophage strains are able to reduce the incidence of bacterial spot caused by *Xanthomonas campestris* pv. *vesicatoria* in tomato by levels equivalent to those obtained by treatments of the traditional copper and mancozeb bactericides. Additionally, bacteriophage treatments increased the yield of tomato plants by 25% compared to those treated with copper and mancozeb, possibly due to the reduced phytotoxicity of the bacteriophage mixture compared with copper. While resistance of bacteria to the bacteriophage did develop, new bacteriophage mutants with restored lytic ability could be generated in the laboratory (Harbaugh et al., 1998).

The use of bacteriophage in the control of fire blight, an aerial rather than soil-borne pathogen, presents additional challenges. Primary among these is the ability of bacteriophage to maintain high populations on the aerial portions of trees. Many studies have noted that bacteriophages may be stabilized by the presence of organic matter or the charged particles found in soil (Burge and Enkiri, 1978; Williams et al., 1987) and tend to be sensitive to both dessication and ultraviolet light (Adams, 1959; Ritchie, 1978).

A mixture of three bacteriophage isolates effectively lysed *E. amylovora* populations *in vitro*, a result not obtained by the phage isolates individually (Schnabel et al., 1999; Palmer et al., 1997). Applications of this mixture to blossoms in the field reduced disease incidence by 26% to 37% (Schnabel et al., 1999). The apparent synergistic effect of multiple bacteriophage mixtures has been attributed to the presumed inability of a single bacterium to simultaneously mutate in three separate loci which would confer resistance.

1.7.3 Bacteriophages of *E. amylovora*

One of the bacteriophages commonly isolated by the Michigan State University group is a species first isolated by Ritchie and Klos (1976), designated PEa1. In subsequent characterizations of this phage (Ritchie and Klos, 1979, Ritchie, 1978), it was reported that PEa1 exhibited a distinctive, large plaque morphology with an expanding, translucent halo. This halo has since been attributed to a capsular depolymerase generated in excess during the lytic cycle of the bacteriophage (Hartung et al., 1988). Ritchie (1978) noted a rapid rise in resistance to PEa1 *in vitro*, but the lack of persistence in this resistance indicated that the lack of continued lytic activity was attributable to the rise in the concentration of capsular depolymerase in the culture, which eliminated PEa1's primary binding site.

Other bacteriophages which bind to bacterial capsular material, such as PK1A which is able to use the capsulated *E. coli* strain K1 as a host, require the capsule for attachment to the surface of the cell and are less able to successfully lyse cells which lack a capsule (Pelkonen, 1992).

1.8 Objectives

The need for effective control measures for fire blight will only increase in the future, as antibiotic resistant strains of the pathogen continue to arise in field populations and commercial fruit growers move towards dense plantings of highly susceptible apple scions (such as Gala, Fuji and Jonagold) grafted to susceptible rootstocks (M.9 or M.26) (Johnson and Stockwell, 1998; Thomas and Jones, 1992; Steiner, 1998). The goals of this project were to isolate and characterize bacteriophages of *E. amylovora* using both traditional and molecular techniques in order to provide insight into the distributions, relatedness and characteristics of these phages. Phages were initially isolated following incubation with a mixture of six *E. amylovora* strains in order to avoid selection of phages based on their affinity for certain strains. Origins of these strains are shown in Table 1. Such multiple host enrichment is more likely to promote the isolation of phages with a broad host range, a desirable characteristic in a biological control agent (Jensen et al., 1998).

The second objective was to evaluate bacteriophage isolates for their potential as biological control agents against *E. amylovora*. Bioassays on model systems of forced pear blossoms and immature pear fruit were used to evaluate the ability of the phages to control *E. amylovora* populations.

2 Materials and Methods

2.1 Media and culture conditions

Strains of *E. amylovora* and *P. agglomerans* were cultured in 9 cm Petri plates containing 2.3% nutrient agar (Difco Laboratories, Detroit, MI), 0.25% yeast extract (Difco) and 0.5% food grade sucrose (NASYE) and incubated at 26 °C for 18-20 hours prior to use. Liquid culture was conducted in 250 ml tissue culture flasks containing 60 ml 0.8% nutrient broth (Difco), 0.25% yeast extract (Difco) and 0.5% food grade sucrose (NBSYE) and incubated at 26 °C in an orbital shaker at 160 rpm for 18-20 hours. Strains of *Pseudomonas* spp. were grown on Pseudomonas agar F (Difco) and incubated at 26 °C for 18-20 hours prior to use. *E. coli* was grown on Luria-Bertani medium (LB) (Difco) and incubated at 37 °C for 18-20 hours prior to use.

Unless otherwise stated, bacteria used in experiments were aseptically scraped from the plate surface, suspended in sterile 10 mM sodium phosphate buffer pH 6.8 (PB), and stored on ice prior to use. Bacterial strains underwent long-term storage in storage medium composed of 0.8% nutrient broth (Difco), 0.25% yeast extract (Difco) and 0.5% food grade sucrose, 0.25% K_2HPO_4, 0.05% KH_2PO_4, 0.025% $MgSO_4$ and 50% (v/v) glycerol and held at -70 °C.

Bacteriophages were plated on 9 cm Petri plates using the soft agar overlay method fully described by Adams (1959). The top agar consisted of 0.8% nutrient agar (Difco), 0.25% yeast extract (Difco) and 0.5% food grade sucrose, with NAS (2.3% nutrient agar (Difco) and 0.5% food grade sucrose) as the bottom layer. Unless otherwise stated, bacterial lawns were prepared by aseptically adding 10 µl diluted phage lysate to 100 µl of a visibly turbid suspension of the bacterial host strain in a 50 ml glass test tube. After 10 min, 3-5 ml molten top agar (40-50 °C) was added to the

tube and the mixture poured into a plate containing 20-25 ml NAS. Phage were always diluted and stored in sterile 0.4% nutrient broth (Difco) at 4 °C.

2.2 Bacterial and bacteriophage strains

Strains of bacteria used in experiments and their origins are listed in Table 1. Bacteriophage PEa1(h) was obtained from the American Type Culture Collection. *E. amylovora* strains Ea6-4, Ea17-1-1, Ea29-7, EaD-7 and EaG-5 used in immature pear plug bioassay experiments were marked with resistance to the antibiotic rifampicin. Rifampicin resistant mutants were selected by plating 5 x 10^7 CFU *E. amylovora* onto NASYE plates amended with 100 µg/ml rifampicin (Sigma Chemical Co., St. Louis, MO). Resistant colonies were subcultured three times on NASYE amended with 100 µg/ml rifampicin, suspended in storage medium and placed into storage at -70 °C. Stability of the mutations was confirmed by subculturing five times on unamended NASYE and replica plating onto NASYE amended with 100 µg/ml rifampicin. Pathogenicity of the marked strains was determined by inoculation into apple seedlings as described below. Continued sensitivity of the marked strains to bacteriophage was established by titering phage isolates on lawns seeded with either their usual bacterial propagation host or its marked derivative.

Sensitivity to streptomycin of bacterial strains Ea17-1-1 rif^r, 110R, EaD-7 rif^r and EaG-5 rif^r was confirmed by placing an 8 mm Whatman No. 1 filter paper disc (Whatman, Inc., Clifton, NJ) saturated with a 100 µg/ml filter-sterilized solution of streptomycin sulfate (Sigma) onto a lawn of bacteria. Such lawns were prepared by streaking a sterile cotton swab soaked in a visibly turbid bacterial suspension across an NASYE plate. Plates were incubated for 18-20 hours and examined for a zone of inhibition surrounding the paper discs.

Table 1. Bacterial strains, phenotypes and their roles in experimentation.

Designation	Phenotype	Use	Source	Donator
Erwinia amylovora				
Ea6-4		Propagation, host range, blossom bioassay	pear	D. Hunter[a]
Ea17-1-1		Propagation, host range, blossom bioassay	pear	D. Hunter
EaD-7		Propagation, host range, blossom bioassay	pear	D. Hunter
EaG-5		Propagation, host range, blossom bioassay	pear	D. Hunter
Ea 29-7		Propagation, host range, blossom bioassay	apple	A. M. Svircev[a]
110R	rif^r	Propagation, host range, all bioassays	apple	A. L. Jones[b]
Ea6-4 rif	rif^r			This work
Ea17-1-1 rif	rif^r	Pear plug bioassay		This work
EaD-7 rif	rif^r	Pear plug bioassay		This work
EaG-5 rif	rif^r	Pear plug bioassay		This work
Ea 29-7 rif	rif^r			This work
Ea 1-97		Host Range	raspberry	G. Braun[c]
Ea 4-96		Host Range	raspberry	G. Braun
Ea 6-96b		Host Range	raspberry	G. Braun
20A		Host Range	crab apple	K. Bedford[d]
29	str^r	Host Range	pear	K. Bedford
34A		Host Range	apple	K. Bedford
1280	str^r	Host Range	apple	K. Bedford
Pantoea agglomerans				
31420		Host Range		ATCC
49018		Host Range		ATCC
Pseudomonas fluorescens				
A506		Host Range		Plant Health Technologies
Pseudomonas syringae				
MB-4		Host Range		T. Zhou[e]
Escherichia coli				
DH5-α		Host Range		A. Castle[f]

[a]Agriculture and Agri-Food Canada, Southern Crop Protection and Food Research Centre, Vineland Station, ON
[b]Department of Botany and Plant Pathology, Michigan State University, East Lansing, MI
[c]Agriculture and Agri-Food Canada, Kentville Agricultural Centre, Kentville, NS
[d]Agriculture and Agri-Food Canada, Pacific Agri-Food Research Centre, Summerland, BC
[e]Agriculture and Agri-Food Canada, Southern Crop Protection and Food Research Centre, Guelph, ON
[f]Department of Biological Sciences, Brock University, St. Catharines, ON

2.3 Pathogenicity tests

Pathogenicity of *E. amylovora* strains was established by adjusting bacterial suspensions to an OD_{600} of 0.1 (equivalent to 1 $x10^8$ CFU/ml) in PB and inoculating 20 µl of this suspension into the apical tips of 6-8 month old potted apple seedlings (cv. Golden Delicious) approximately 20-40 cm in height and observing the development of disease symptoms after 7-14 d in a 27 °C greenhouse. A disease severity index (Figure 5) was used to rate symptoms with 0 representing no disease and 5 representing severe disease symptoms.

2.4 Phage collection

Collections were made from mid June to late August, 1998 from sites in and around the Niagara Region and Burlington, ON. Cuttings were taken from the aerial portions of trees which exhibited symptoms typical of fire blight and placed into 18x16.5 cm plastic freezer bags with "ziplock" style closures (DowBrands Canada, Paris, ON). Soil samples were taken from the bases of trees with blight symptoms using a stainless steel soil corer, driven to a depth of 10-20 cm approximately 1 m from the base of the tree. Soil samples were also stored in freezer bags. Samples were pooled by site, keeping the aerial and soil samples separate. Cutting instruments were disinfected with 70% ethanol between cuttings in order to minimize transmission of the pathogen. Locations and types of trees collected from are shown in Table 2.

2.5 Sample enrichment

All soil and aerial samples were enriched in a procedure modified from Crosse and Hingorani (1958). Flasks containing 60 ml NBSYE were inoculated with 200 µl of each of the six *E. amylovora* propagation hosts listed in Table 1. Into each flask was

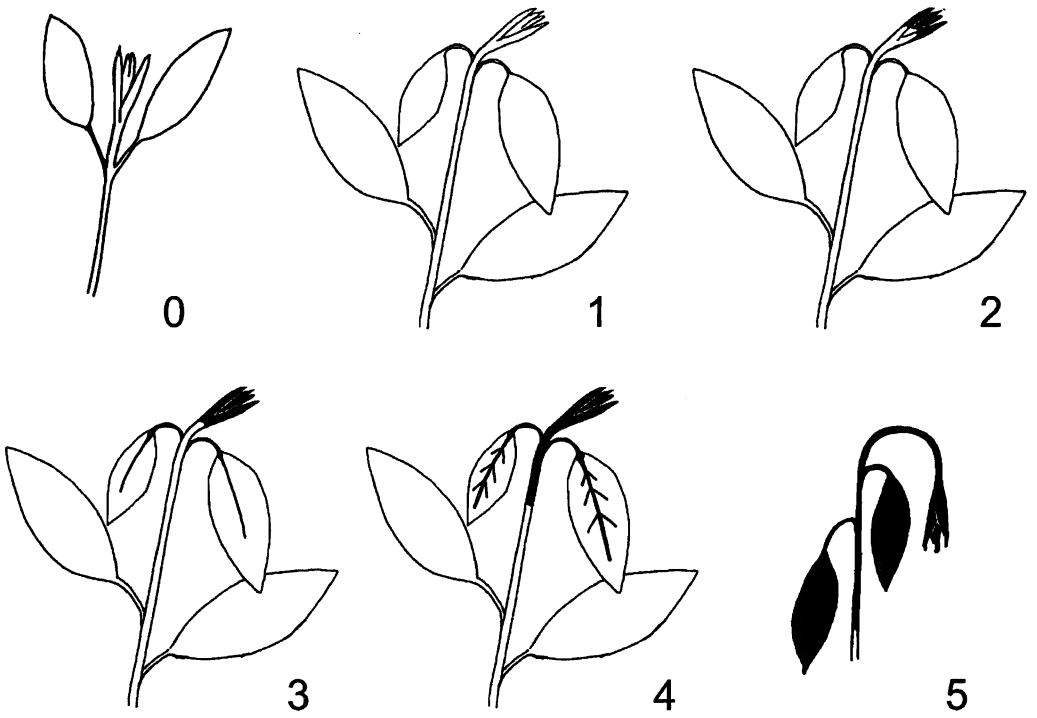

Figure 5. Disease severity index used for determination of pathogenicity of *E. amylovora* isolates in apple seedlings. A rating of 0=no disease, 1=wilting, 2=wilting and blackening of apical tip, 3=blackening extends into central vein of upper leaves, 4=blackening extends into leaves and down into seedling stem, 5=complete blackening of upper leaves and upper stem.

placed 50-60 g wet weight of soil or 10-20 g fresh weight aerial tissue, and incubated for 18 to 20 hr at 26 °C and 160 rpm. The resulting slurry was then decanted into 50 ml round-bottom Teflon centrifuge tubes to which 500 µl chloroform had previously been added. The tubes were agitated thoroughly and centrifuged at 4 °C and 8 000 g for 20 min. The supernatant was removed with a pipette and stored at 4 °C in 50 ml glass test tubes containing 2% (v/v) chloroform.

2.6 Isolation of bacteriophage

Supernatant from the enrichment step was diluted by 10^2, 10^3 and 10^4 times and plated onto six lawns each seeded with one of the propagation hosts listed in Table 1. Lawns were checked for the formation of plaques after 24 and 48 hours. Single plaques were picked from these lawns using sterile Pasteur pipettes and placed into micro centrifuge tubes containing 1 ml NBSYE and 2% (v/v) chloroform. Tubes were centrifuged at 8 000 g and stored at 4 °C. Bacteriophage isolates were purified by passage through this single plaque isolation procedure three times. Once picked from a lawn seeded by a given bacterial strain, that phage isolate was propagated and titered on that strain exclusively.

2.7 Enrichment of bacteriophage isolates

High titer lysates of bacteriophage isolates were obtained by the confluent plate method described by Adams (1959). Confluently lysed plates were flooded with 5 ml of 0.4% nutrient broth (Difco) and agitated gently for 40-60 min on a flatbed orbital shaker. The lysate was removed with a pipette, placed over 2% (v/v) chloroform and centrifuged at 8 000 g for 20 min. The supernatant was removed and stored in 15 ml glass scintillation vials with either Teflon or foil seals containing 2% (v/v) chloroform at 4

Table 2. Locations and collection dates of bacteriophage isolates.

Date Collected	Location[a]	Crop
June 16, 1998	Vineland	Pear (*Pyrus*)
June 16, 1998	Vineland	Apple (*Malus*)
July 6, 1998	Vineland	Pear
July 20, 1998	Vineland	Pear
July 20, 1998	Vineland	Apple
July 27, 1998	Grimsby	Pear
July 27, 1998	Burlington Golf Course, Burlington	Crab Apple (*Malus*)
Aug. 26, 1998	Royal Botanical Gardens, Burlington	Mountain ash (*Sorbus*)
Aug. 26, 1998	Royal Botanical Gardens, Burlington	Magnolia (*Magnolia*)

[a] All sites are located in Ontario, Canada

°C. High titer lysates were always placed on ice when not under refrigeration.

Titers were established by a "spot lysis" technique where 10 µl of each step of a 10-fold serial dilution of lysates were dropped onto a pre-poured lawn consisting of 3-5 ml top agar seeded with 100 µl of a visibly turbid suspension of the phage's host bacteria. Plates were allowed to sit open in a laminar flow bench for 10 min prior to incubation at 26 °C for 18-20 h. In most cases, higher dilutions yielded spots containing a number of individually countable plaques; otherwise, titer was estimated from the dilution yielding the last confluent spot. Phage titers were checked once every 2-4 weeks.

2.8 Plaque morphology

Phage lysates were diluted to produce approximately 50 PFU per plate, and added to 100 µl of bacterial suspensions which were adjusted to 1 x 10^8 CFU/ml. After 10 min, 3.0 ml of molten top agar was added to the tube and poured over NAS. Plates were incubated at 26 °C for 24 h and photographed against a black background with a Hitachi HV-C20M digital camera (Hitachi) controlled by KS 400 version 2.0 digital imaging software (Kontron Elektronik, Eching, Germany). Plaques were compared based on range of plaque diameter, clarity and presence of a translucent halo.

2.9 Host range analysis

The host ranges of all phage isolates were tested against 13 bacterial strains representing *E. amylovora*. Host ranges of a limited number of phages was also tested against five bacterial strains representing four species other than *E. amylovora*. Bacterial strains used in host range experiments are listed in Table 1. Lawns of bacterial strains were prepared by seeding 3.0 ml top agar with 100 µl of bacterial

suspensions adjusted to 1 x 10^8 CFU/ml. Phage lysates were diluted to a concentration of 1 x 10^7 PFU/ml and 10 µl of this dilution was spotted onto the solidified lawns.

Plates were dried open in a laminar flow hood for 10 min and incubated at 26 °C for 18-20 hr. Areas of visible clearing under points of phage application were scored as positive, while areas which looked no different that the surrounding untreated lawn were scored as negative. Experiments were repeated three times.

2.10 Pear blossom bioassays

Blossoms harvested from susceptible cultivars provide a reliable model system for evaluating the performance of potential biological control agents against *E. amylovora*. Shoots bearing dormant blossom buds, 40-60 cm in length, were collected from field-grown pear trees (cv. Flemish Beauty and Bartlett) in January-March of 1999 from Victoria Farm in Vineland Station, ON (University of Guelph) and a local commercial orchard. Cuttings were used immediately or bundled and stored at 4 °C prior to use.

Branches were surface disinfected by dipping the cut ends in 70% ethanol for 2-3 min and rinsing thoroughly with tap water. The branches were placed into buckets containing tap water to a depth of 15-20 cm and incubated in a Conviron model PGW36 controlled environment cabinet (Conviron Controlled Environments Inc., Winnipeg, MB) at 22 °C and 70% RH for 12-15 d, or until the appearance of the blossoms at the "popcorn" stage of development. Water in the buckets was changed every 3-5 d in order to control microbial growth.

Glass scintillation vials with plastic screw caps into which holes had previously been drilled with a 3/16" bit were filled with tap water. Recently opened blossoms with a healthy appearance were harvested by hand and individually placed into the vials

such that the peduncle of the blossom extended through the hole and into the water contained in the vial. Vials containing blossoms were stored in plastic racks inside closed plastic bins (Tupperware) and stored for a maximum of 5 d at 4 °C.

Bacterial suspensions were adjusted to 1 x 10^8 CFU/ml and applied to the blossoms "to run off" using 40 ml plastic pump aerosol sprayers in a spray booth. Blossoms were allowed to air dry (approximately 3 h) and were then inoculated with a suspension of 1 x 10^7 PFU/ml phage in PB containing 1 mM $MgSO_4$ and 100 µg/ml gelatin in the same manner. All phage isolates were tested only against their bacterial propagation hosts. For each bacterial host, blossoms inoculated with bacteria and sterile buffer served as a positive control. Uninoculated blossoms and blossoms inoculated with PB or PB amended with 1 mM $MgSO_4$ and 100 µg/ml gelatin alone served as negative controls. All treatments contained 5 blossoms and were arranged in blocks in the racks.

Vial racks were returned to the plastic bins and incubated in the controlled environment cabinet at 22 °C and 70% RH. Blossoms were evaluated after 3 d using a rating scale modified from Pusey (1997) which is shown in Figure 6.

In later experiments, blossoms were inoculated with bacterial and phage suspensions using an adjustable pipettor. Blossoms were inoculated with 10 µl of pathogen suspension followed immediately by 10 µl of phage suspension. All other conditions were followed as described above.

2.11 Immature pear plug bioassays

Cores of immature pear fruit have been used in evaluating potential biological control agents (Vanneste et al., 1995). Pear plugs were used in this work to evaluate the biological control activity of phage isolates by screening for the presence or

absence of bacterial exudate (ooze), and also by quantifying the bacterial population remaining on the plug surface after treatment with phage.

Immature pear fruits (*Pyrus communis* L. cv. Bartlett) were collected from a commercial orchard (Bill Moyer, Grimsby, ON) in late July 1999 and stored at 1 °C until use. When excessive ripening of these fruits in cold storage became apparent, immature fruit was collected again in early September, 1999 from the same location. Pears were surface disinfected by soaking in 70% ethanol for 2 min and aseptically sliced latitudinally to a thickness of 2-4 mm. Plugs of a diameter of 11 mm were punched from the slices using a sterilized cork borer, avoiding the skin and the pith of the fruit. Plugs were placed into a beaker and mixed. A minimum of five fruits were used in each experiment in order to randomize variations caused by individual differences between fruits.

Bacteriophage suspensions were always made and inoculated on the same day in order to ensure accurate titers. Phage were suspended in PB amended with 7 mM $MgSO_4$. In all cases phage isolates were only evaluated against their corresponding bacterial propagation host. One treatment for each of the bacterial strains was bacteria plus sterile PB amended with 7 mM $MgSO_4$, which served as a positive control. Plugs inoculated with PB and PB amended with 7 mM $MgSO_4$ served as negative controls. All treatments contained 5 plugs. Plugs with the same treatment were placed in five separate Petri dishes.

Plugs were placed on 9 cm qualitative Whatman No. 1 filter paper discs moistened with tap water in 9 cm diameter Petri plates. The plates containing inoculated plugs were incubated at 27 °C in closed plastic bins lined with wet paper towels to maintain a high RH.

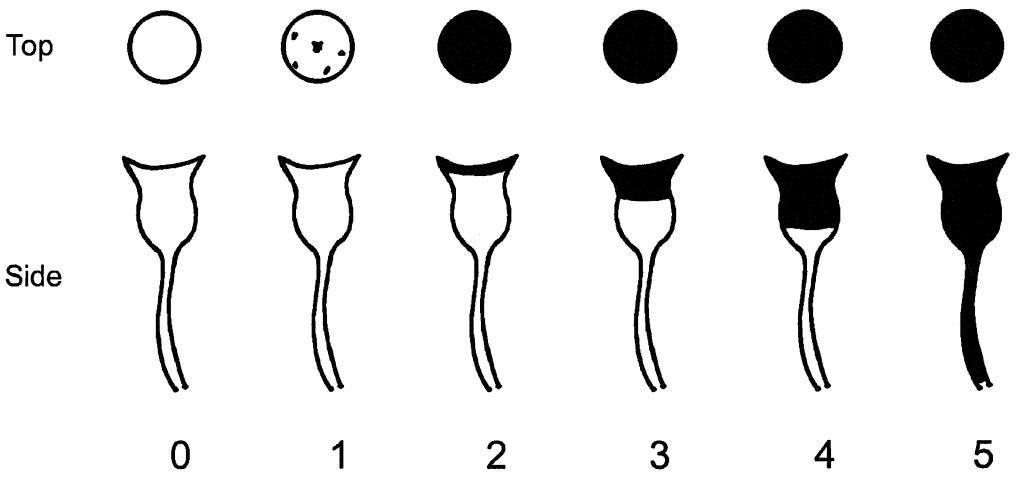

Figure 6. Disease severity index used in the evaluation of forced pear blossoms in bioassay experiments. A rating of 0=no necrosis, 1=incomplete necrosis of the hypanthium, 2=complete necrosis of hypanthium, 3=hypanthium and up to one-half of ovary necrotic, 4=hypanthium and between one-half and whole ovary necrotic, 5=necrosis extending below ovary. Rating scale adapted from Pusey (1997).

2.11.1 Evaluation of exudate development

Plugs were inoculated with 10 µl of bacterial suspensions adjusted to 1 x 10^8 CFU/ml followed immediately by 10 µl of 1 x 10^8 PFU/ml suspensions of bacteriophage, resulting in a multiplicity of infection (MOI) of 1. The plugs were incubated for 42 hours and evaluated for the presence or absence of macroscopically visible ooze on the plug surface. Plugs exhibiting any visible exudate ("ooze") on the surface (as compared with the uninoculated controls) were scored as positive. The experiment was repeated four times. Data was pooled and analyzed statistically with Z-tests for two proportions pairwise against the control treatment using SigmaStat version 2.0 (Jandel Scientific, San Rafael, CA).

2.11.2 Quantification of bacteria surviving phage treatment

Six phage isolates were selected on the basis of their performance in the plaque morphology, host range and pear plug bioassay screening experiments for further study in experiments to quantify the number of viable *E. amylovora* cells remaining on the plug surface after treatment with phage.

In the first set of experimental conditions, 10 µl of a 1 x 10^8 CFU/ml bacterial suspension was inoculated onto the plug surface followed immediately by 10 µl of a 1 x 10^8 PFU/ml suspension of phage (MOI = 1). This experiment was repeated three times. In a second experiment, plugs were inoculated with 10 µl of a 1 x 10^7 CFU/ml bacterial suspension followed immediately by 10 µl of a 1 x 10^8 PFU/ml suspension of phage (MOI = 10). This experiment was repeated three times. For all quantification experiments, plugs were inoculated with rifampicin-resistant derivatives of the bacterial propagation host strains. All treatments contained five replicates.

To determine the relative efficacy of the currently recommended chemical control

measure for fire blight, plugs were inoculated with 10 µl of a 1 x 10^8 CFU/ml suspension of bacteria followed immediately by 10 µl of a 200 µg/ml solution of streptomycin sulfate (Sigma) in water. This 1:1 dilution of the antibiotic resulted in a final concentration of 100 µg/ml on the plug surface, which is the current recommended application rate.

All plugs were incubated in closed plastic bins in the same manner as described previously. Each plug was added to a separate sterile 50 ml glass test tube containing 10 ml sterile PB, vortexed and placed in a sonication bath for 30 s (Cavitator, Mettler Electronics Corp., Anaheim, CA). Tubes were then vortexed again and placed on ice. The resulting suspensions were serially diluted in sterile PB and plated onto NASYE amended with 50 µg/ml rifampicin and 50 µg/ml cycloheximide to control fungal growth. Colonies were counted after 24-48 h incubation.

Data from each experiment were analyzed by Mann-Whitney rank sum tests pairwise against the control treatment using SigmaStat version 2.0 (Jandel Scientific). The number of CFU present on each phage or antibiotic treated plug was calculated and this number divided by the mean CFU present on positive control plugs inoculated with the same bacterial strain in the same replicate of the experiment. This provided a value of percent growth relative to the control.

2.12 Transmission electron microscopy

Phage lysate was dialysed against distilled water at 4 °C overnight. One drop of lysate was placed onto a 200 x 200 mesh nickel grid coated with formvar and allowed to sit for 2 minutes, followed by one drop of 5% uranyl acetate applied without wicking off the lysate. This was allowed to sit for 3 minutes before the solution was wicked off and the grid dried. Specimens were viewed using a Philips CM10 transmission electron microscope with an accelerating voltage of 80 kV.

2.13 Molecular analysis

2.13.1 DNA extraction

Phage isolates were enriched in 5 ml NBSYE liquid cultures contained in 50 ml glass test tubes. Two tubes for each isolate (a total culture volume of 10 ml) were inoculated with 50 µl of bacterial suspension and 10 µl of phage lysate containing 10^7 to 10^{10} PFU/ml. Cultures were incubated for 18-20 h at 25 ºC and 220 rpm in an orbital shaker. All phage isolates were enriched using *E. amylovora* strain 110R as host. Lysates were prepared using chloroform and centrifugation steps as described previously, with the exception that lysates were not stored over chloroform after centrifugation.

Phage DNA was extracted using a method modified from Manfioletti and Schneider (1988). To each 10 ml volume of lysate was added DNase I (Boehringer Mannheim, Laval, QC) to a final concentration of 20 µg/ml and RNase (Boehringer Mannheim) to a final concentration of 100 µg/ml. After incubation at room temperature for 15 min, 0.8 ml of 0.5 M EDTA (Sigma) pH 8 and proteinase K (Boehringer Mannheim) to a final concentration of 50 µg/ml were added, and incubated at 45 ºC for 15 min. DNA was precipitated in the presence of 0.2% (w/v) hexadecyl trimethyl ammonium bromide (CTAB) (Sigma) and 20 mM NaCl and incubated at 65 ºC for 3 min, followed by cooling on ice and pelleting of the DNA:CTAB complex in a centrifuge at 8 000 g for 10 min. The pellet was resuspended in a minimal volume (usually 1 ml) of 1.2 M NaCl and the DNA precipitated with two volumes of 95% ethanol. DNA was resuspended in a minimal volume of sterile distilled water and stored at -20 ºC. Concentrations of DNA yield were estimated by running in a 0.8% agarose gel (Promega Corp., Madison, WI) in TAE buffer (Tris 4.84 g/l, glacial acetic acid 1.14 ml/l, EDTA 0.001 M) and comparing to a 1kb DNA ladder (MBI Fermentas, Flamborough,

ON).

2.13.2 Restriction fragment length polymorphisms

Restriction endonuclease digestions were conducted using enzymes *Eco*R I (recognition sequence 5' GAATTC 3'), *Bgl* II (5' AGATCT 3'), *Bam*H I (5' GGATCC 3') (all from Promega) and *Tha* I (5' CGCG 3') (Life Technologies, Rockville, MD) according to the supplier's instructions and using 0.5 to 1 µg of DNA, 3 units enzyme and 0.1 mg/ml acetylated BSA per 50 µl reaction. Digestion of each enzyme/phage DNA combination was conducted in a separate tube. Samples were digested with *Eco*R I, *Bgl* II and *Bam*H I overnight at 37 °C, and with *Tha* I for 1-2 h at 60 °C. Fragments were run on a 1% agarose gel (Promega) in TAE and stained with 1 µg/ml ethidium bromide.

2.13.3 Polymerase chain reaction

Primer sequences specific for the bacteriophage PEa1 were generously donated by A. L. Jones of Michigan State University. Primer sequences were 5' AATGGGC ACCGTAAGCAGT 3' for PEa1-A and 5' TAATGGGTATGATAGAAGGCAGAC 3' for PEa1-B. Primers were expected to amplify a 304 bp product.

Reactions were run in 50 µl volumes using 0.2 µM of each primer PEa1-A and PEa1-B (Norgen Biotek, St. Catharines, ON), 1X PCR buffer, 0.2 mM of each dNTP, 1.5 mM $MgCl_2$ and 1.5 units of *Taq* polymerase (MBI Fermentas). One µl of a 1 x 10^8 PFU/ml phage suspension in PB was used as template. Reactions were run in a Perkin Elmer GeneAmp 9600 thermocycler (Perkin Elmer, Norwalk, CN) under the following conditions: 95 °C, 2 min; 95 °C, 30 s, 53 °C, 30 s, 72 °C, 30 s, 30 cycles.

3 Results

3.1 Pathogenicity tests

E. amylovora strains isolated from apple or pear produced typical fire blight symptoms when inoculated into apple seedlings. Mean symptom severity ranged from 2.0 for strain Ea29-7 to 5.0 for strains Ea6-4, 110R, EaG-5, 34a, 20a and 29 (Table 3). Rifampicin resistant derivatives of *E. amylovora* strains produced symptoms in apple seedlings, while strains isolated from raspberry caused little or no symptom development.

3.2 Bacteriophage isolation

A total of 54 bacteriophage isolates were collected between June and September of 1998. The majority, 45 isolates, were collected from soil samples while only nine phages were isolated from aerial tissue. Attempts at isolating phages at three separate sites from in or around trees which did not exhibit fire blight symptoms were unsuccessful.

Ten of the bacteriophage isolates collected in 1998 were lost during the initial purification procedure or after extended storage. The 45 phage isolates used in this study (including phage PEa1) are described in Table 4. A map (Figure 7) illustrates the locations of phage collections. Titers of these isolates were generally stable when stored at 4 °C and handled on ice.

Table 3. Severity of fire blight disease symptoms induced in potted Golden Delicious apple seedlings by *E. amylovora* stains and their rifampicin-resistant derivatives.

Bacterial strain	Disease index[a]
Ea 6-4	5.0
Ea 6-4 rif	5.0
Ea 17-1-1	3.7
Ea 17-1-1 rif	4.3
Ea D-7	4.3
Ea D-7 rif	3.7
Ea G-5	5.0
Ea G-5 rif	5.0
Ea 29-7	2.0
Ea 29-7 rif	3.7
Ea 110R	5.0
Ea 1-97	0.0
Ea 4-96	0.0
Ea 6-96b	1.0
34a	5.0
20a	5.0
1280	4.7
29	5.0
Water control	0.0

[a]Disease rating of 0=no disease, 1=wilting, 2=wilting and blackening of apical tip, 3=blackening extends into central vein of upper leaves, 4=blackening extends into leaves and down into seedling stem, 5=complete blackening of upper leaves and upper stem.

Table 4. Groupings, locations and dates of collection of *E. amylovora* bacteriophage isolates.

			E. amylovora	Source of phage		
Group[a]	Isolate	Date collected	host	Site[b]	Crop	Aerial/Soil
1	Eram1	June 16, 1998	Ea 110	2	Pear	soil
	Eram2	June 16, 1998	Ea G-5	2	Pear	soil
	Eram3	June 16, 1998	Ea D-7	2	Pear	soil
	Eram4	June 16, 1998	Ea 6-4	2	Pear	soil
	Eram5	June 16, 1998	Ea 110	1	Apple	soil
	Eram16	July 20, 1998	Ea 6-4	4	Apple	aerial
	Eram17	July 20, 1998	Ea D-7	4	Apple	aerial
	Eram18	July 20, 1998	Ea G-5	4	Apple	aerial
2	Eram6	June 16, 1998	Ea D-7	1	Apple	soil
	Eram8	June 16, 1998	Ea 29-7	1	Apple	soil
	Eram9	July 6, 1998	Ea 17-1-1	3	Pear	soil
	Eram11	July 6, 1998	Ea 110	3	Pear	soil
	Eram13	July 6, 1998	Ea D-7	3	Pear	soil
	Eram14	July 6, 1998	Ea 29-7	3	Pear	soil
	Eram20	July 20, 1998	Ea 29-7	4	Apple	aerial
3a	Eram7	June 16, 1998	Ea 29-7	1	Apple	soil
	Eram21	July 20, 1998	Ea 29-7	4	Apple	soil
	Eram22	July 20, 1998	Ea 29-7	4	Apple	soil
	Eram23	July 20, 1998	Ea 110	4	Apple	soil
	Eram24	July 20, 1998	Ea 110	4	Apple	soil
	Eram25	July 20, 1998	Ea 17-1-1	4	Apple	soil
	Eram29	July 20, 1998	Ea 110	4	Apple	soil
3b	Eram41	July 27, 1998	Ea D-7	6	Crab Apple	aerial
3c	PEa1	Summer 1975	Ea 110	ATCC	Apple	aerial
4	Eram10	July 6, 1998	Ea 110	3	Pear	soil
	Eram12	July 6, 1998	Ea D-7	3	Pear	soil
	Eram26	July 20, 1998	Ea G-5	4	Apple	soil
	Eram30	July 20, 1998	Ea 17-1-1	4	Apple	soil
5	Eram31	July 20, 1998	Ea 17-1-1	5	Pear	soil
	Eram33	July 20, 1998	Ea G-5	5	Pear	soil
	Eram34	July 20, 1998	Ea 6-4	5	Pear	soil
6	Eram44	Aug. 26, 1998	Ea 17-1-1	8	Magnolia	soil
	Eram45	Aug. 26, 1998	Ea 110	8	Magnolia	soil
	Eram46	Aug. 26, 1998	Ea 6-4	8	Magnolia	soil
	Eram47	Aug. 26, 1998	Ea 29-7	8	Magnolia	soil
	Eram49	Aug. 26, 1998	Ea 6-4	8	Magnolia	soil
	Eram50	Aug. 26, 1998	Ea 29-7	8	Magnolia	soil
Ungrouped	Eram15	July 20, 1998	Ea 17-1-1	4	Apple	aerial
	Eram19	July 20, 1998	Ea 110	4	Apple	aerial
	Eram27	July 20, 1998	Ea 6-4	4	Apple	soil
	Eram28	July 20, 1998	Ea 110	4	Apple	soil
	Eram32	July 20, 1998	Ea 29-7	5	Pear	soil
	Eram35	July 27, 1998	Ea 29-7	7	Pear	soil
	Eram37	July 27, 1998	Ea G-5	7	Pear	soil
	Eram51	Aug. 26, 1998	Ea 17-1-1	8	Magnolia	soil

[a]Groups based on molecular markers, described in sections 3.3 and 3.4.
[b]Physical locations of sites are shown in Figure 7.

3.3 Polymerase chain reaction

Using the primers specific for bacteriophage PEa1, phages Eram7, Eram21, Eram22, Eram23, Eram24, Eram25, Eram27, Eram28, Eram 29 and Eram41 produced a ca. 300 bp PCR product, indicating relatedness to phage PEa1 (Figure 8). Phage PEa1 obtained from the ATCC also produced this fragment.

3.4 Restriction fragment length polymorphisms

DNA was extracted from the 44 surviving bacteriophage isolates collected from the field and phage PEa1. Of these 45 isolates, 37 were placed into one of six groups based on the patterns obtained by digestion of the bacteriophage DNA with four restriction endonucleases. The remaining eight isolates showed patterns which were a combination of two other restriction patterns, and were assumed to be the result of cross-contaminated cultures. Isolates Eram27 and Eram28, for instance, which were identified as PEa1-type phages using PCR, produced patterns which were a combination of group 3 (PEa1 type) and group 4 phages (Figure 10). The arrangement of phage isolates into groups based on RFLP data is shown in Table 4; characteristics of these restriction patterns are shown in Table 5. Agarose gel electrophoresis patterns of phage DNA representative of these groups are shown in Figure 9.

All of the phages which were identified as related to PEa1 using PCR produced similar restriction patterns. These phages were placed into group 3, with the exceptions of Eram27 and Eram28 noted above. Unlike all other molecular groups, however, phages in group 3 were further divided into three subgroups, based on variations in restriction patterns. Isolates Eram7, Eram21, Eram22, Eram23, Eram24, Eram25 and Eram29 produced identical restriction patterns and were designated as group 3a. Phage Eram41, the only surviving phage isolated from the aerial portion of a crab apple

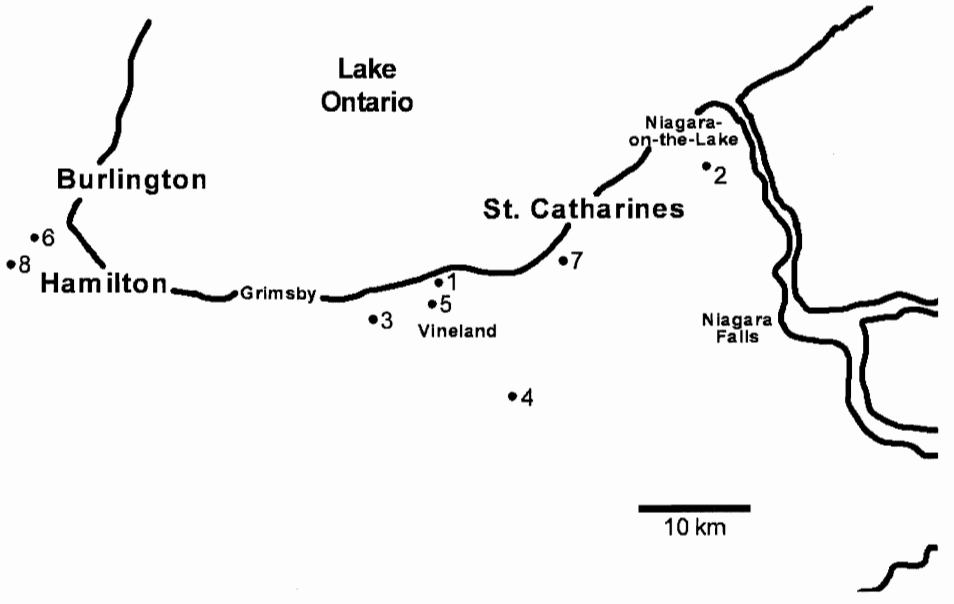

Figure 7. A silhouette map of the Niagara Region depicting general locations of sites from which bacteriophages of *E. amylovora* were successfully isolated.

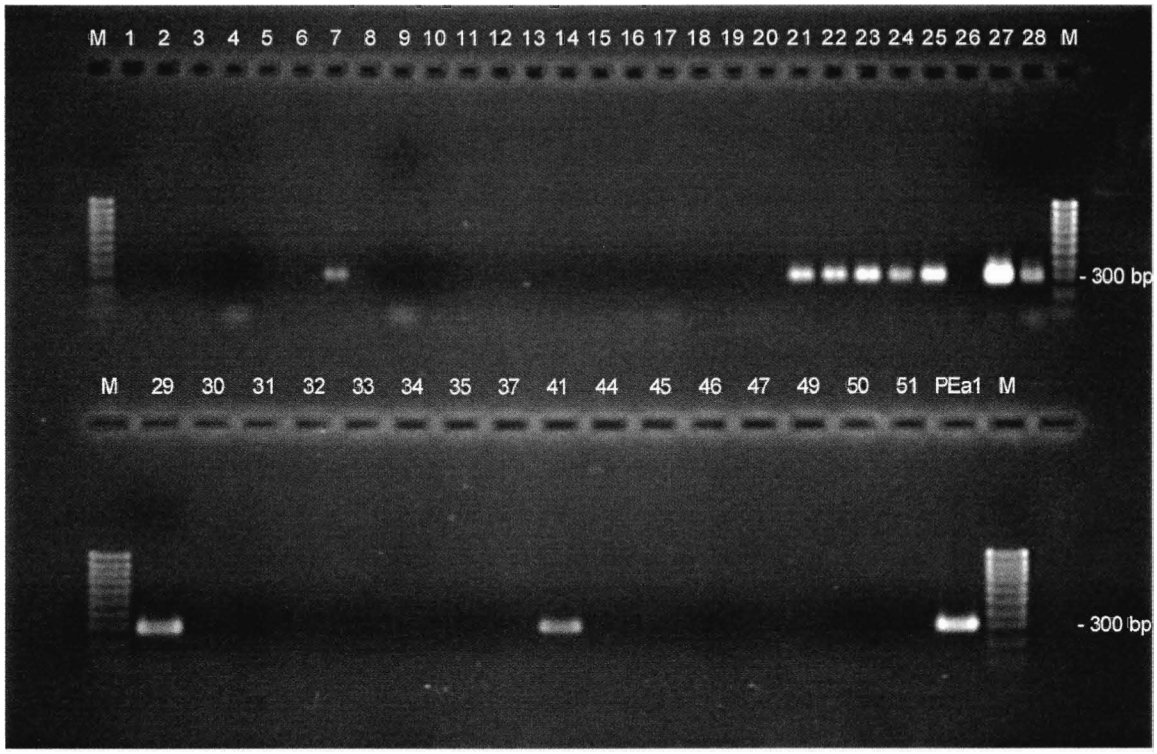

Figure 8. Agarose gel electrophoresis of PCR products amplified using primers PEa1-A and PEa1-B, designed to amplify a ca. 300 bp product from phage PEa1. M: 100 bp DNA ladder as size standard (MBI Fermentas); lane numbers indicate corresponding Eram phage isolate; PEa1: product amplified from phage PEa1 as positive control.

Table 5. Bacteriophage isolates placed into groups based on digestion of phage DNA with four restriction endonucleases.

Group	Restriction endonuclease	Number of fragments produced[a]	Estimated genome size (kbp)[b]
1	*Eco*R I	17	73
	Bgl II	1	—[c]
	*Bam*H I	1	—
	Tha I	21	54
2	*Eco*R I	1	—
	Bgl II	18	74
	*Bam*H I	1	—
	Tha I	>30	—
3a	*Eco*R I	4	—
	Bgl II	8	36
	*Bam*H I	1	—
	Tha I	11	14
3b	*Eco*R I	1	—
	Bgl II	10	44
	*Bam*H I	1	—
	Tha I	11	14
3c	*Eco*R I	1	—
	Bgl II	9	37
	*Bam*H I	1	—
	Tha I	11	14
4	*Eco*R I	15	67
	Bgl II	1	—
	*Bam*H I	1	—
	Tha I	20	46
5	*Eco*R I	11	—
	Bgl II	19	43
	*Bam*H I	1	—
	Tha I	16	42
6	*Eco*R I	1	—
	Bgl II	17	62
	*Bam*H I	21	52
	Tha I	9	—

[a]Fragments as resolvable by agarose gel electrophoresis in 1.0% and 1.8% agarose slab gels.
[b]Genome length estimated by summing lengths of resolvable fragments.
[c]Fragment sizes larger than 15-20 kbp cannot be reliably estimated.

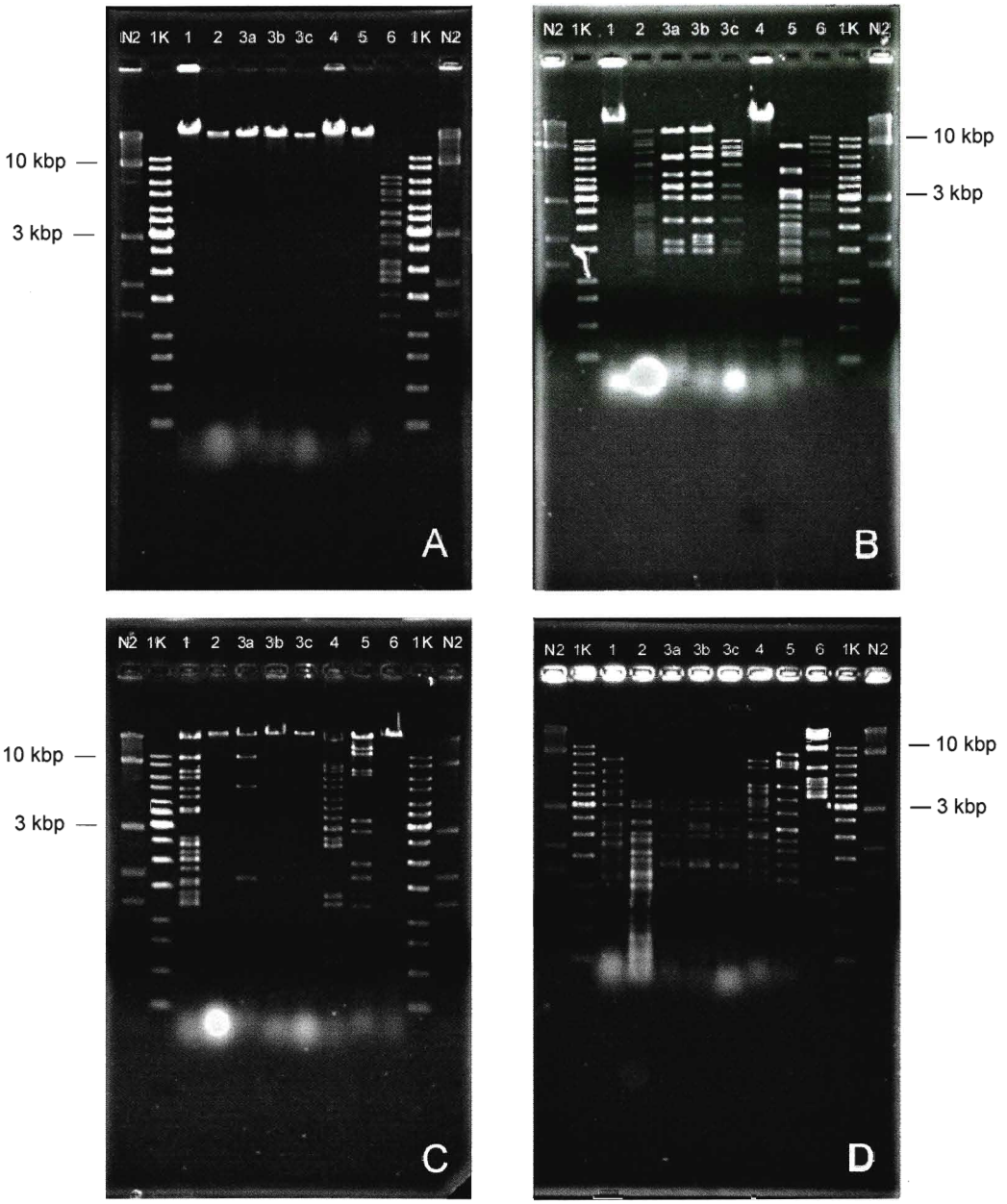

Figure 9. Agarose gel electrophoresis of restriction patterns representative of those exhibited by six phage groups. A: digestion with *Bam*H I; B: digestion with *Bgl* II; C: digestion with *Eco*R I; D: digestion with *Tha* I. Individual lanes are labeled as follows: N2: N2 DNA size standard (Norgen Biotek, St. Catharines, ON); 1K: 1 Kb DNA ladder (MBI Fermentas); labels 1, 2, 3a, 3b, 3c, 4, 5, and 6 indicate the phage group which the pattern represents.

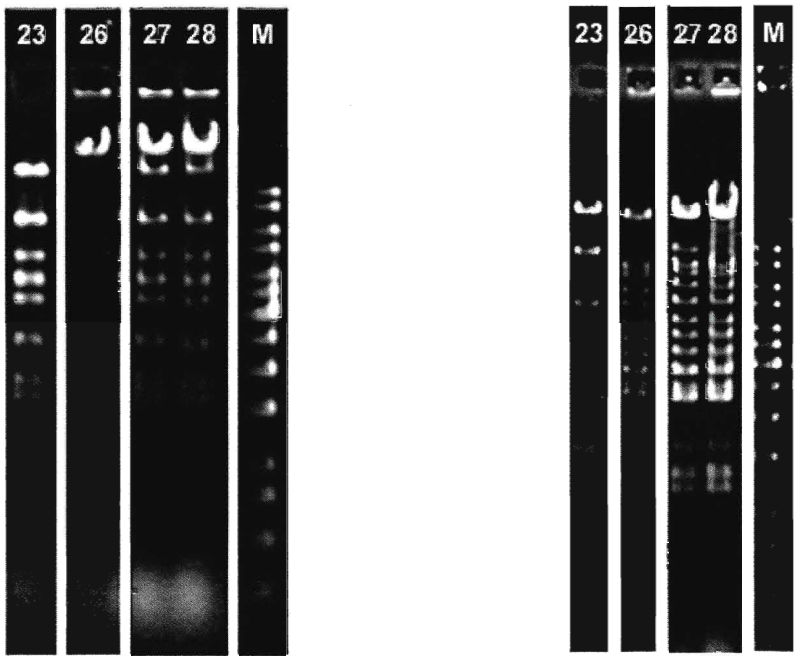

Figure 10. An example of the restriction patterns produced by phage isolates in the "ungrouped" category when digested with two enzymes. Lanes marked 23 are isolate Eram23, from group 3a; lanes marked 26 are isolate Eram26, from group 4; lanes marked 27 and 28 are isolates Eram27 and Eram28, which display additive restriction patterns from the phages from groups 3a and 4, suggestive of cross-contamination. Isolates which produced similar phenomenoa were placed into the "ungrouped" category as shown in Table 4. M: 1 Kb DNA ladder (MBI Fermentas).

tree, produced two more bands than the phages in group 3a when digested with endonuclease *Bgl* II (Fig. 9B), 2 different bands when digested with *Tha* I (Fig. 9D) and was undigested by *Eco*R I (Fig. 9C); Eram41 was placed into group 3b. Phage PEa1, isolated in 1976 in Michigan, was also undigested by *Eco*R I and produced two fragments which were of differing lengths from those found in group 3a when digested with *Bgl* II (Fig 9B), and one extra fragment when digested with *Tha* I (Fig. 9D). Phage PEa1 was placed into group 3c.

Restriction endonuclease *Bam*H I did not cleave DNA of any phage isolates except for those placed into group 6, which were all isolated from the Royal Botanical Gardens. Phages in this group all exhibited identical restriction patterns when digested with this enzyme, producing 21 fragments.

DNA was extracted from phages Eram2, Eram4, Eram46 and Eram47 after growth on their own bacterial host strains as listed in Table 4. The restriction patterns of phages Eram2, Eram4 and Eram47 were identical to those produced by digestion of DNA from these phages when grown on bacterial strain 110R. The DNA of bacteriophage Eram46, however, was uncut by endonucleases *Bam*H I and *Bgl* II; when grown on strain 110R, the DNA of this phage was cut into 21 and 17 fragments by these enzymes, respectively.

3.5 Plaque morphology

Bacteriophage isolates collected in the summer of 1998 produced one of four general plaque types on lawns of *E. amylovora* (Figure 11). Those phages identified as related to phage PEa1 by PCR and RFLP analysis (groups 3a, 3b and 3c) produced distinctive 4-6 mm plaques with a translucent halo which continued to expand after the plaque itself stopped growing. A second plaque type, produced by phages placed into

groups 2 and 4, was much smaller, approximately 0.5 mm in diameter, with an expanding translucent halo. Phages in groups 1 and 5 produced plaques with a diameter of 1-3 mm with an undefined, or ragged edge and no halo. The fourth plaque type, produced by phages in group 6, was 1-2 mm in diameter with a defined edge and no halo. Plaque clarity varied slightly among phages in the same molecular group.

3.6 Transmission electron microscopy

Phage isolate Eram2 was stained and viewed under the transmission electron microscope. Phage Eram2 (Figure 12) possesses a hexagonal (presumably icosahedral) head with a long, contractile tail.

3.7 Host Range

Of the 37 phages evaluated, 15 (40%) were able to produce visible plaques on all 13 *E. amylovora* strains tested. When arranged by the groupings determined by the phages' molecular characteristics, two groups, 3 and 6, exhibited characteristic host range patterns. Host ranges of phage groups 1-6 are shown in Tables 6-11, respectively. Host range patterns do not appear to correlate to the bacterial strain used to isolate and propagate the phage (Table 12). The host ranges of the eight ungrouped phages are not considered here as phage mixtures would exhibit additive host range patterns and would therefore appear artificially broad. Phages in group 3, (Table 8) PEa1 and its relatives, showed little or no lytic activity against *E. amylovora* strains 29, 34A and 1280, all isolated from British Columbia orchards, and against strains EaG-5 and Ea6-4, isolated from Harrow, Ontario. The exception to this pattern was phage Eram7, which formed plaques on all *E. amylovora* strains.

In group 6 phages (Table 11), the same pattern of apparent host resistance was

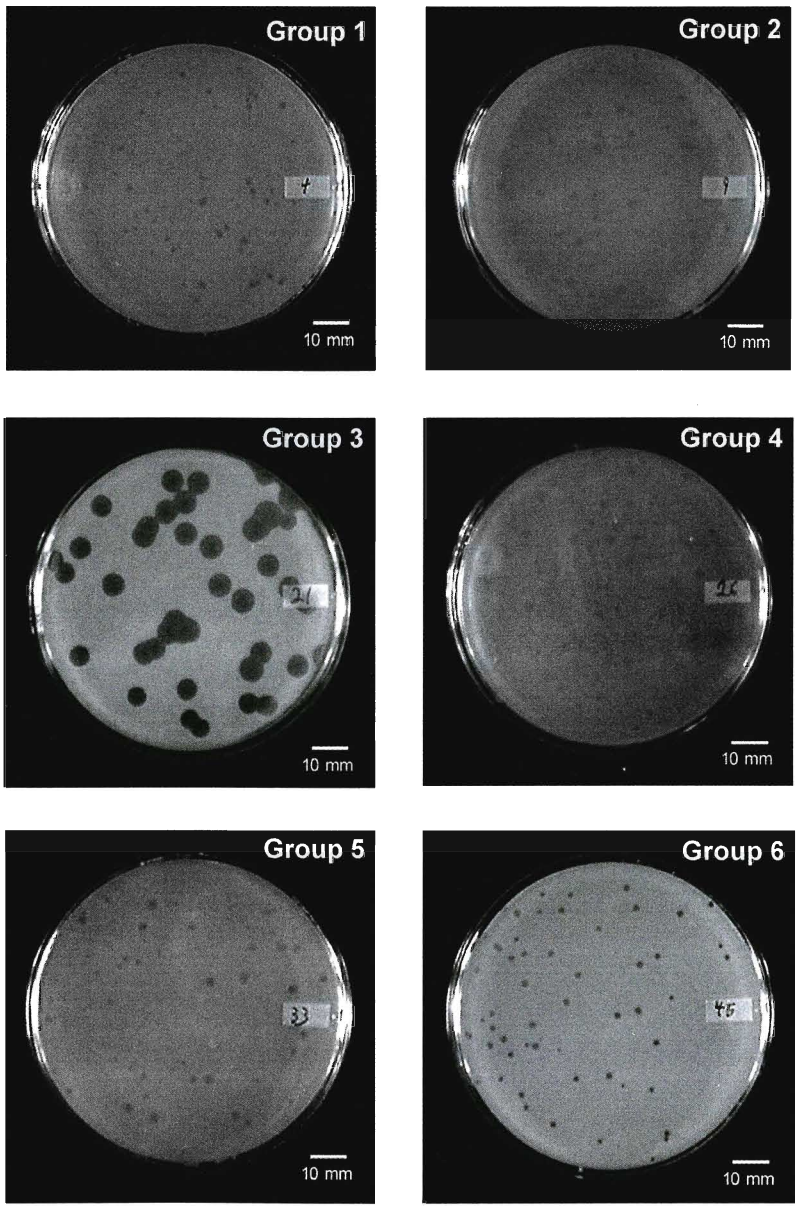

Figure 11. Plaques formed by phages on lawns of their own *E. amylovora* host strain using the soft agar overlay method. Note the plaques formed by phages in groups 1 and 5 are similar in appearance, as are those formed by phages in groups 2 and 4.

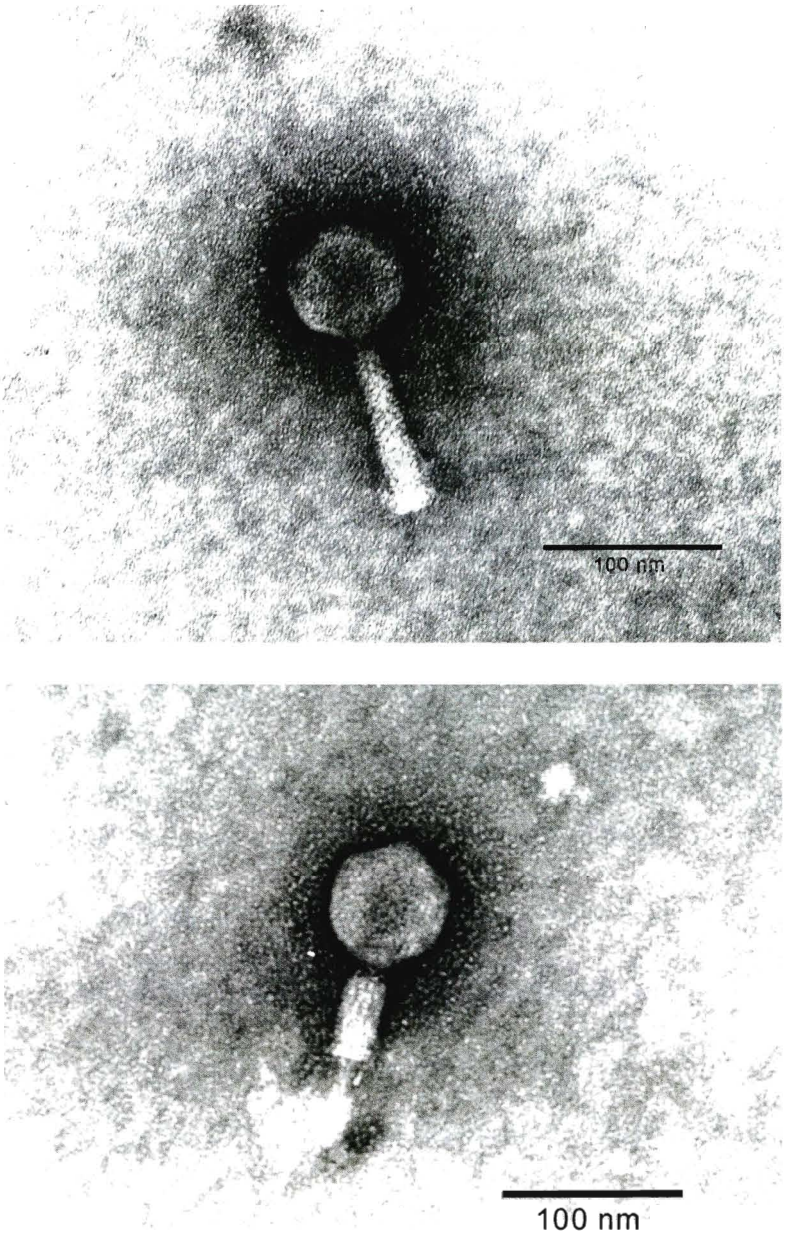

Figure 12. Transmission electron micrographs of phage Eram2. Top: intact virion. Bottom: phage with a contracted tail, exposing inner tail core. x280 000.

evident, with the addition of bacterial strain Ea4-96 which also appeared unlysed by these phages. The exceptions to this pattern were phages Eram46, which lysed all *E. amylovora* strains, and Eram49, which produced plaques on strains EaG-5, Ea6-4, 29, 34A and 1280, but did not produce plaques on strain Ea29-7; Eram49 was the only phage isolate unable to lyse this bacterial strain.

The phages in group 5 (Table 10) also produced weak or no clearing on lawns of *E. amylovora* 34A and 1280. One phage in this group, Eram33, failed to produce plaques on *E. amylovora* 29. Host range patterns of other phage groups were generally broad; some intra-group variation is apparent, particularly in the phages' ability to produce plaques on lawns of *E. amylovora* strains 34A, 1280, Ea6-4 and EaG-5.

A limited number of isolates were selected for evaluation of their ability to form plaques on lawns composed of bacterial species other than *E. amylovora* (Table 13). Of the 14 isolates used in these experiments, none were able to lyse lawns composed of *E. coli* DH5-α, *P. syringae* MB-4 or *P. fluorescens* A506,(BlightBan A506, Plant Health Technologies). The two phage isolates from group 1 tested were both able to produce plaques on both *P. agglomerans* strains evaluated. None of the five isolates evaluated from group 3a produced plaques on these *P. agglomerans* strains, but the related phage Eram41, placed into group 3b, was able to produce plaques on *P. agglomerans* 49018. Of the six members of group 6, five were tested and all were able to lyse *P. agglomerans* 49018; two of these phages, Eram47 and Eram50, were also able to produce weak plaques on *P. agglomerans* 31420.

3.8 Pear blossom bioassay

Cuttings of blossom bud-bearing material taken from the field produced healthy blossoms when forced in early 1999. Experiments conducted in January and February

Table 6. Host range patterns of phages in group 1. Boxed cells indicate bacterial strain on which the phage was isolated and propagated.

	Phage isolate							
E. amylovora strain	Eram1	Eram2	Eram3	Eram4	Eram5	Eram16	Eram17	Eram18
G-5	+a	+	-	+	-	+	+	+
D-7	+	+	+	+	+	+	+	+
6-4	+	+	+	+	+	+	+	+
17-1-1	+	+	+	+	+	+	+	+
Ea 4-96	-b	NT	+	+	+	(+)	+	NT
Ea 6-96b	(+)c	NT	+	+	+	+	+	+
Ea 1-97	+	+	+	+	+	+	+	+
Ea 29-7	NTd	(+)	+	+	+	(+)	(+)	(+)
20A	NT	+	+	+	+	+	+	+
29	+	+	+	+	+	+	+	+
34A	(+)	NT	+	(+)	+	(+)	+	NT
1280	(+)	(+)	+	(+)	+	(+)	NT	(+)
110R	+	+	+	+	+	+	+	+

[a]Plus sign indicates plaque formation.
[b]Minus indicates no plaque formation.
[c]Indicates weak plaque formation.
[d]Not tested.

Table 7. Host range patterns of phages in group 2. Boxed cells indicate bacterial strain on which the phage was isolated and propagated.

	Phage isolate						
E. amylovora strain	Eram6	Eram8	Eram9	Eram11	Eram13	Eram14	Eram20
G-5	-[a]	-	(+)	-	+	+	+
D-7	+[b]	+	+	+	+	+	+
6-4	+	+	+	+	+	+	+
17-1-1	+	+	+	+	+	+	+
Ea 4-96	+	+	+	+	+	+	+
Ea 6-96b	+	+	+	+	+	+	+
Ea 1-97	+	+	+	+	+	+	+
Ea 29-7	+	+	+	+	+	+	+
20A	+	+	+	+	+	+	+
29	+	+	+	+	+	+	+
34A	(+)c	+	+	+	+	+	+
1280	+	+	+	+	+	+	+
110R	+	+	+	+	+	+	+

[a]Minus indicates no plaque formation.
[b]Plus sign indicates plaque formation.
[c]Indicates weak plaque formation.

Table 8. Host range patterns of phages in group 3. Boxed cells indicate bacterial strain on which the phage was isolated and propagated.

	Phage isolate								
	Group 3a							Group 3b	Group 3c
E. amylovora strain	Eram7	Eram21	Eram22	Eram23	Eram24	Eram25	Eram29	Eram41	PEa1
G-5	+[a]	(+)[b]	(+)	(+)	(+)	(+)	(+)	-	NT
D-7	+	+	+	+	+	+	+	**+**	+
6-4	+	(+)	(+)	(+)	-	-	(+)	-	-
17-1-1	+	+	+	+	+	**+**	+	+	NT
Ea 4-96	+	(+)	(+)	NT[d]	NT	+	NT	+	+
Ea 6-96b	+	+	+	+	+	+	+	+	NT
Ea 1-97	+	+	+	+	+	+	+	+	+
Ea 29-7	**+**	**+**	**+**	+	+	+	+	+	+
20A	+	+	+	+	+	+	+	+	+
29	+	-[c]	-	-	-	-	-	-	-
34A	+	-	-	-	-	-	-	-	-
1280	+	-	-	-	-	-	(+)	-	-
110R	+	+	+	**+**	**+**	+	**+**	+	**+**

[a]Plus sign indicates plaque formation.
[b]Indicates weak plaque formation.
[c]Minus indicates no plaque formation.
[d]Not tested.

Table 9. Host range patterns of phages in group 4. Boxed cells indicate bacterial strain on which the phage was isolated and propagated.

	Phage isolate			
E. amylovora strain	Eram10	Eram12	Eram26	Eram30
G-5	+[a]	+	+	+
D-7	+	+	+	+
6-4	+	+	+	+
17-1-1	+	+	+	+
Ea 4-96	+	NT[b]	NT	-[d]
Ea 6-96b	+	+	+	+
Ea 1-97	+	+	+	+
Ea 29-7	+	+	(+)	+
20A	+	+	+	+
29	+	+	+	+
34A	+	(+)[c]	+	(+)
1280	+	(+)	(+)	(+)
110R	+	+	+	+

[a]Plus sign indicates plaque formation.
[b]Not tested.
[c]Indicates weak plaque formation.
[d]Minus indicates no plaque formation.

Table 10. Host range patterns of phages in group 5. Boxed cells indicate bacterial strain on which the phage was isolated and propagated.

	Phage isolate		
E. amylovora strain	Eram31	Eram33	Eram34
G-5	+[a]	**+**	+
D-7	+	+	+
6-4	+	+	**+**
17-1-1	**+**	+	+
Ea 4-96	NT[b]	(+)[d]	(+)
Ea 6-96b	+	+	+
Ea 1-97	+	+	+
Ea 29-7	+	+	+
20A	+	+	+
29	+	-	+
34A	-c	(+)	(+)
1280	-	-	(+)
110R	+	+	+

[a]Plus sign indicates plaque formation.
[b]Not tested.
[c]Minus indicates no plaque formation.
[d]Indicates weak plaque formation.

Table 11. Host range patterns of phages in group 6. Boxed cells indicate bacterial strain on which the phage was isolated and propagated.

	Phage isolate					
E. amylovora strain	Eram44	Eram45	Eram46	Eram47	Eram49	Eram50
G-5	(+)[a]	(+)	(+)	(+)	**+**	(+)
D-7	+[b]	+	+	+	(+)	+
6-4	-[c]	-	**+**	-	+	-
17-1-1	**+**	+	+	+	+	+
Ea 4-96	-	-	+	-	-	-
Ea 6-96b	+	+	+	+	+	+
Ea 1-97	+	+	+	+	+	+
Ea 29-7	+	+	+	**+**	-	**+**
20A	+	+	+	+	+	+
29	-	-	+	-	+	-
34A	-	-	+	-	(+)	-
1280	-	-	+	-	(+)	(+)
110R	+	**+**	+	+	+	+

[a]Indicates weak plaque formation.
[b]Plus sign indicates plaque formation.
[c]Minus indicates no plaque formation.

Table 12. Host range patterns of phages which were isolated and propagated on *E. amylovora* strain Ea29-7. Note how patterns tend to be conserved within groups based on molecular markers.

	Phage isolate							
	Group 2			Group 3a			Group 6	
E. amylovora strain	Eram8	Eram14	Eram20	Eram7	Eram21	Eram22	Eram47	Eram50
G-5	-[a]	+	+	+	(+)[c]	(+)	(+)	(+)
D-7	+	+	+	+	+	+	+	+
6-4	+	+	+	+	(+)	(+)	-	-
17-1-1	+	+	+	+	+	+	+	+
Ea 4-96	+	+	+	+	(+)	(+)	-	-
Ea 6-96b	+	+	+	+	+	+	+	+
Ea 1-97	+	+	+	+	+	+	+	+
Ea 29-7	+	+	+	+	+	+	+	+
20A	+	+	+	+	+	+	+	+
29	+	+	+	+	-	-	-	-
34A	+	+	+	+	-	-	-	-
1280	+	+	+	+	-	-	-	(+)
110R	+	+	+	+	+	+	+	+

[a]Minus indicates no plaque formation.
[b]Plus sign indicates plaque formation.
[c]Indicates weak plaque formation.

Table 13. Host range patterns of selected phages on bacterial species other than *E. amylovora*.

	Phage isolate													
	Group 1		Group 3a					Group 3b	Group 4	Group 6				
Bacterial Strain	Eram2	Eram18	Eram7	Eram22	Eram23	Eram24	Eram29	Eram41	Eram26	Eram44	Eram45	Eram46	Eram47	Eram50
E. coli DH5-alpha	-[a]	-	-	-	-	-	-	-	-	-	-	-	-	-
P. agglomerans 49018	+[b]	+	-	-	-	-	-	+	-	+	+	+	+	+
P. agglomerans 31420	+	+	-	-	-	-	-	-	+	-	-	-	(+)c	(+)
P. syringae MB-4	-	-	-	-	-	-	-	-	-	-	-	-	-	-
P. fluorescens A506	-	-	-	-	-	-	-	-	-	-	-	-	-	-

[a]Minus indicates no plaque formation.
[b]Plus sign indicates plaque formation.
[c]Indicates weak plaque formation.

of 1999 indicated that some phage isolates inhibited fire blight symptom development in detached pear blossoms. These experiments could not be repeated, however, due to the deterioration of blossom quality as time progressed. Blossoms used in experiments in March and April of 1999 often opened unevenly or experienced petal fall shortly after opening, and displayed an overall unhealthy, wilted appearance. When inoculated with *E. amylovora*, these blossoms showed uneven symptom development or additional symptoms atypical of fire blight. It is due to this unreliability that data collected from pear blossom bioassays are not presented here.

3.9 Immature pear plug bioassay

3.9.1 Evaluation of exudate production

Twenty-three bacteriophage isolates exhibited significant biological control activity in the immature pear plug system when evaluated for their ability to inhibit the development of symptoms in the form of bacterial exudate or "ooze". When arranged by RFLP group (Figure 13), the phages in groups 3 and 6 exhibited the highest levels of overall biological control activity. The ungrouped isolates Eram28, Eram35 and Eram51 also controlled ooze production. In the case of a few isolates, such as Eram2, variations in biocontrol activity between trials were evident, however the majority of phages produced consistent control activity across all trials. The majority of isolates in groups 1, 2, 4 and 5 tested using this system exhibited minimal biological control activity.

3.9.2 Quantification of bacteria surviving phage treatment

Based on their performance in this initial screening and on differences exhibited in the host range and plaque morphology analysis, six phage isolates were selected

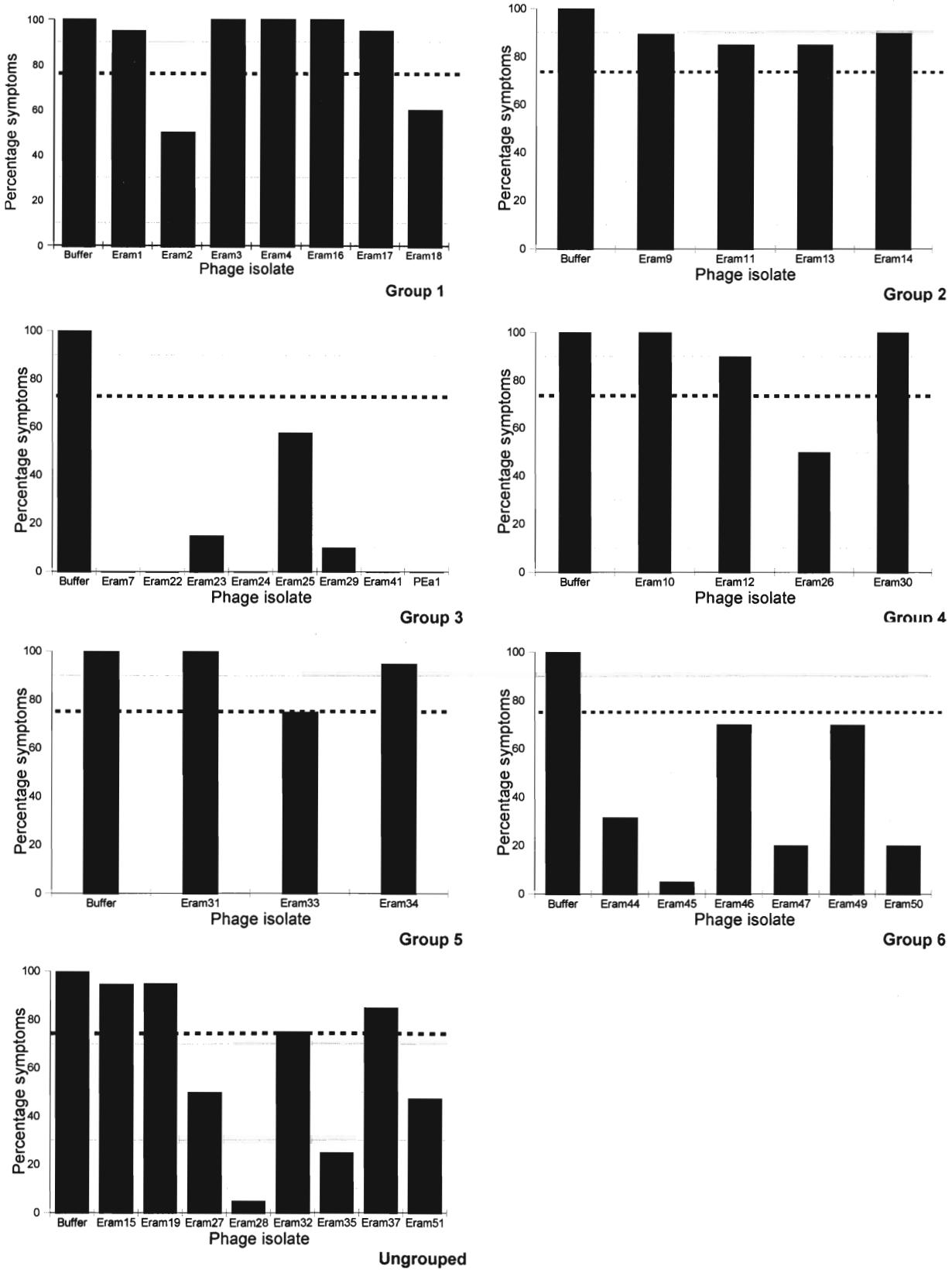

Figure 13. Ability of bacteriophages to control exudate production on plugs of immature pear fruit. Percentages represent proportions of 20 inoculated plugs across four experiments showing fire blight symptoms. Phage isolates are arranged by groups as determined by molecular markers. Dashed line indicates significant control, P=0.05.

for further study in the pear plug system. The absence of visible ooze on the pear plug surface did not preclude the possibility that a sizeable population of *E. amylovora* was not resident on the surface of the immature pear plug.

Phages Eram2, Eram24, Eram26, Eram41, Eram45 and Eram51 were initially applied at an MOI of 1 (Figure 14). Due to the variability expressed by this system, statistical significance at $P \leq 0.05$ was not achieved with all phage isolates in all trials; in the third and final trial, no phages were able to give significant control when $P \leq 0.05$. Phages Eram2, Eram26 and Eram45 exhibited the strongest biological control ability, with significant control in both trials 1 and 2. Phage Eram51 did not provide significant control in any trial. The biological control level exhibited by Eram41 in trial 1 is considered an outlier.

In the absence of a control agent, the bacterial population on the plug surface increased by 100 fold or more, from 1×10^6 CFU at the time of application to between 1×10^8 CFU and 5×10^{10} CFU at the time of evaluation. Phage treatment was able to reduce this population increase by as much as 97% in the case of phage Eram45 in trial 2 (Fig. 14). Significant reductions, however, represent a bacterial population of 6×10^7 to 2×10^9 CFU still resident on the plug surface after phage treatment. In a second set of experiments, the concentration of phage relative to the pathogen was increased 10 fold in order to determine if control activity could be enhanced.

The three trials conducted at an MOI of 1 used immature fruit harvested in July 1999. Due to the excessive ripening of the fruit in storage, experiments conducted at an MOI of 10 used pear fruit harvested in early September, 1999 (Figure 15). While significant control ($P \leq 0.05$) was obtained in some instances, the overall effectiveness of the phages did not increase greatly over the control ability observed when the MOI was 1.

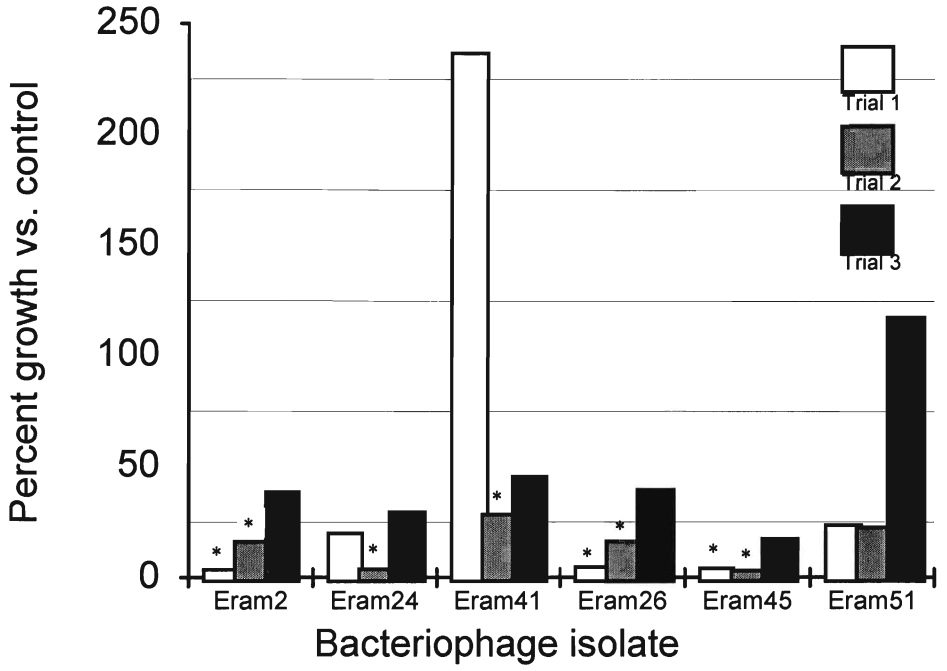

Figure 14. Ability of bacteriophage isolates to control populations of *E. amylovora* on the surface of immature pear fruit in three replicate trials when phage:pathogen ratio (MOI) is 1. Data are presented as the mean CFU recovered from phage treated plugs divided by the mean CFU recovered from control plugs treated with pathogen alone. Statistically significant control ($P \leq 0.05$) is indicated by an asterisk (*).

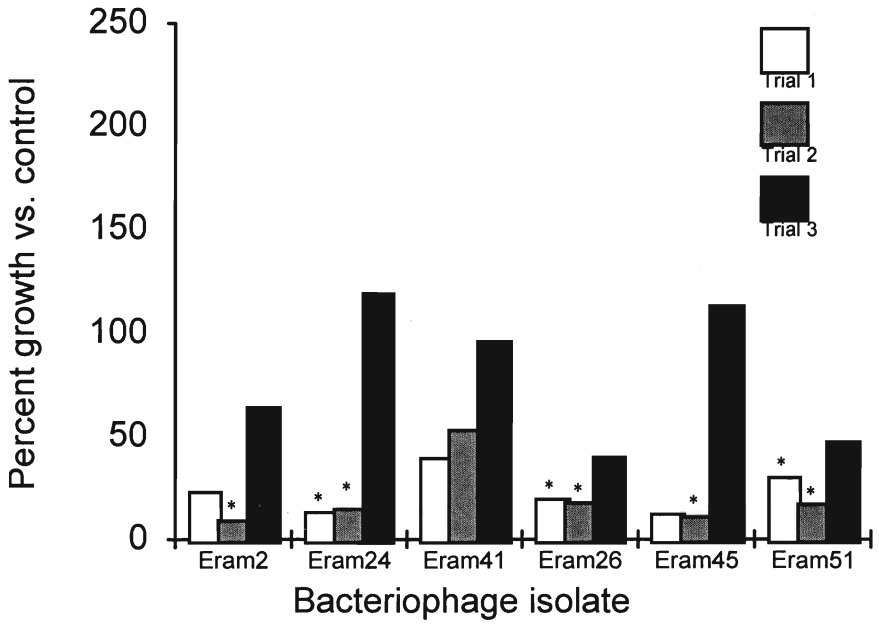

Figure 15. Ability of bacteriophage isolates to control populations of *E. amylovora* on the surface of immature pear fruit in three replicate trials when phage:pathogen ratio (MOI) is 10. Data are presented as the mean CFU recovered from phage treated plugs divided by the mean CFU recovered from control plugs treated with pathogen alone. Statistically significant control ($P \leq 0.05$) is indicated by an asterisk (*).

When streptomycin sulfate at a concentration of 100 µg/ml was evaluated for its ability to control bacterial growth in the pear plug system, significant control was not achieved against any of the four pathogen strains used (Figure 16).

Bacterial colonies which were susceptible to phage were easily recoverable from pear plugs inoculated with phage and pathogen. These colonies grew on medium amended with rifampicin, indicating that they possessed the rif^{r} phenotype. These bacterial isolates were not characterized further for their similarity to the strains originally deposited on the pear plug surface.

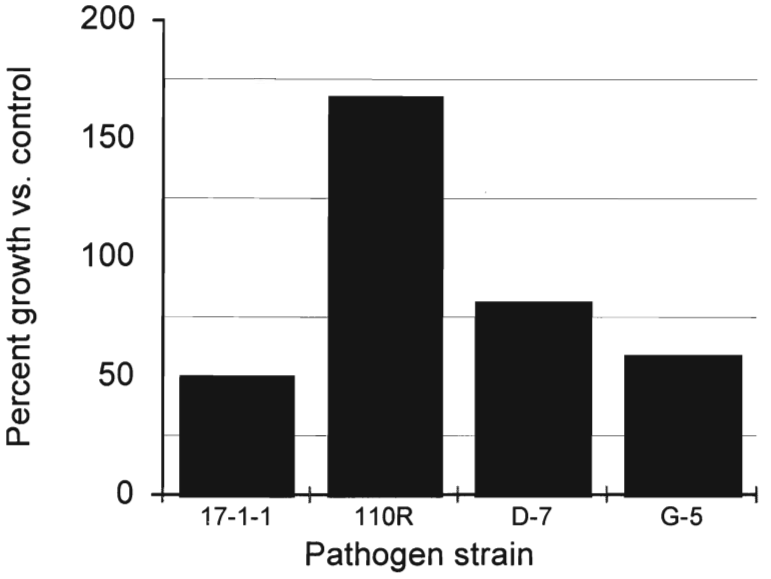

Figure 16. Ability of 100 µg/ml streptomycin sulfate to control populations of *E. amylovora* on the surface of immature pear fruit. Data is presented as the mean CFU recovered from antibiotic treated plugs divided by the mean CFU recovered from control plugs treated with pathogen alone. Statistically significant control ($P \leq 0.05$) was not obtained.

4 Discussion

4.1 Phage isolation and characterization

Of the 44 phages collected from the field in the summer of 1998, 37 were isolated from soil (Table 4). Only seven phages were isolated from aerial environments, despite the fact that aerial samples exhibiting fire blight symptoms were consistently collected and enriched. This finding is in agreement with that of Erskine (1973), who isolated phages from the soil surrounding infected trees and not from aerial samples. In contrast, Ritchie (1976, 1978) was able to easily isolate phages from diseased aerial tissue in Michigan apple orchards.

Phages are amphoteric in nature, carrying a net negative charge at pH values found in most soil environments (Burge and Enkiri, 1978). Phages have been shown to be stabilized in soil environments due to adsorption to charged colloids such as clays (Williams et al., 1987). Soil provides protection against dessication and ultraviolet light, two factors which will inactivate a wide variety of phages, including those of *E. amylovora* (Adams, 1959; Erskine, 1973; Ritchie, 1978). Viable *E. amylovora* phages could be recovered from soil samples collected in 1998 after 12 months storage at 4 °C, demonstrating this stability in soil (data not shown).

In contrast, aerial plant surfaces, while offering an abundance of the host bacterium, provide little in the way of protection against environmental stresses. Phages of *E. amylovora* isolated by Ritchie (1978) were able to tolerate temperature fluctuations but not dessication or ultraviolet light; he postulated that the phages may survive in hydrated environments in the tree canopy. It is unlikely that populations of *E. amylovora* phages are able to reside permanently in aerial tissues, as Ritchie (1978) was unable to isolate *E. amylovora* phages from aerial tissue in the winter months.

The fact that phages were not recoverable from locations which were not proximal to trees exhibiting fire blight symptoms suggests that the presence of the pathogen is required for phage populations to increase to levels detectable by the isolation procedures used. Phage multiplication may occur when stray *E. amylovora* cells are carried into the soil by rain, wind or by infected plant material which detaches from the tree. Conversely, phage may be carried into the tree canopy while adsorbed to wind-blown dust particles from the orchard floor or by insects which visit both environments. Differences in ground cover, precipitation and wind conditions at various sites may therefore affect the distribution of *E. amylovora* phages between the soil and aerial environments.

Several of the phages tested were able to infect the epiphytic bacterium *P. agglomerans* (Table 12), which is commonly found in association with *E. amylovora*. A phage of *E. amylovora* isolated by Erskine (1973) was also able to infect and lysogenize an unidentified yellow epiphytic bacterium, which may have been a *P. agglomerans* strain. The population of the phages of *E. amylovora* may therefore be partly dependent on populations of *P. agglomerans* which act as possible secondary hosts. It was postulated by Erskine (1973) that such epiphytic bacteria may act as a reservoir of prophage in nature. The lysogenic ability of the *E. amylovora* phages collected in this work was not examined.

Incubation of field samples with a mixture of six *E. amylovora* host strains was employed specifically to prevent selection of phages based on affinity for various bacterial strains and to increase the diversity of the collection. An examination of the host ranges (Tables 6-11) indicates that a number of the phages in the collection may not have been isolated had a single bacterial strain been used exclusively for phage isolation. The use of chloroform in these procedures may have contributed to the loss

of some isolates as phage sensitivity to chloroform, previously thought to be a rare phenomenon (Adams, 1959) is now thought to be more common (Ackermann and DuBow, 1987a). Phages which are not easily cultured or maintained are not particularly desirable as potential biological control agents, as the ability to raise and store the phage on a large scale would be a necessary requirement for its field use (Schisler and Slininger, 1997).

The use of restriction endonucleases to characterize the phages on the molecular level proved to be a useful tool in organizing phages in the collection. Phages were placed into six groups based on restriction patterns (Table 4), with one group, group 3, subdivided into three subgroups. The phages of group 3 were all identifiable as PEa1 type by both the PCR protocol and by their production of distinctive, large plaques with expanding haloes. The majority of these phages, placed into group 3a, produced identical restriction patterns which differed slightly from their type phage, PEa1, and from phage Eram41 (Fig. 9).

Phage PEa1 was isolated in 1975 from a Michigan apple orchard, and so it is not surprising that a certain amount of divergent evolution has occurred between two phages separated by such a distance in time and space. Phage Eram41, the sole isolate in group 3b, was isolated approximately 70 km from the phages of group 3a and also exibits slight differences in its restriction patterns, It is interesting to note that this phage appears to be resident across North America, as other workers have isolated PEa1-type phages from Michigan and California (A. L. Jones, personal communication)

As can be seen in Table 4, many of the phages grouped together based on restriction patterns were isolated from the same site. The eight phages in group 1, for instance, were collected from three sites; the six inhabitants of group 6 were all isolated from the same location. In these cases, it is not unreasonable to make the assumption

that phage isolates exhibiting identical restriction patterns and which were isolated from the same sample are probably the same phage, isolated multiple times on different bacterial strains. This assumption cannot be made regarding phages which exhibit identical restriction patterns isolated from separate sites, as genetic divergences not detectable using the restriction endonucleases used may exist between phages resident as isolated populations. Phages with identical restriction patterns can be said to be highly related to each other regardless of site of isolation. The groups based on molecular markers will aid in streamlining future research as representative phages from these groups may be selected for trials.

Alteration in restriction patterns produced by passaging phage Eram46 through various *E. amylovora* strains is suggestive of the presence of a DNA modification system in strain Ea6-4. Modification of Eram46 DNA by this strain would also explain why this phage exhibits a host range which is broader than other members of its group, as shown in Table 11. Enzymes *Bam*H I, *Bgl* II and *Eco*R I will not cleave at their recognition sequences if ^{m5}C is present in the target sequence (Nelson and McClelland, 1991). It is tempting to suggest that this is evidence of a previously uncharacterized DNA modification system in this isolate of *E. amylovora*. However, passing phage Eram4 through the same bacterial strain did not produce any alteration in the phages' restriction pattern; further work must be conducted before presence of a modification system can be conclusively determined.

A number of the phages produced distinctive plaque morphologies when plated on lawns of *E. amylovora* using the soft-agar overlay technique. Figure 1 demonstrates the plaques formed by group 3 phages are large and easily distinguished from those produced by other phages. In this case, plaque morphology proved a reliable indicator of phage relatedness as determined by molecular markers. The plaques produced by

other phages were of limited value in determining relationships between phages in the collection. The phages of groups 2 and 4 produced highly similar plaques as did those of groups 1 and 5, suggesting a similarity which is not borne out by molecular analysis. The plaques produced by phages of group 6 were more distinctive, although differences in plaque clarity between phages of this group grown on different hosts occasionally made classification difficult. Plaque morphology is influenced by the replication rates of the host bacterium and phage and rate of phage diffusion through the medium (Mandell and Eisenstark, 1953). In this manner unrelated phages which share similar characteristics may therefore produce similar plaques.

Transmission electron microscopy of phage Eram2 indicated that this isolate possessed an icosahedral head with a contractile tail. This places Eram2 into phage morphogroup A1 based on the system devised by Bradley (1965) and refined by Ackermann and DuBow (1987a). This also places this phage into the virus order *Caudovirales*, family *Myoviridae*. Phages of this family possess a linear, double-stranded DNA genome. This phage bears a pysical appearance which is similar to that of *P. agglomerans* phage Y46/(E2) (Harrison and Gibbins, 1975).

Overall, the bacterial strain used for isolation and propagation of phages did not impart characteristic host ranges to phage isolates independently of the phages' group. Segregation of host range patterns based on molecular groupings revealed that particular phage types exhibit characteristic host range patterns which are generally not affected by the strain of the propagation host. Phages in groups 3 and 6 (Tables 8 and 11, respectively), with few exceptions, were unable to infect certain bacterial strains at the phage titer applied. Of the limited number of phages evaluated, several were able to infect *P. agglomerans* stains (Table 12), a facet of the host range analysis which also assorted by molecular group. None of the phages tested were able to lyse *P.*

fluorescens A506 (BlightBan, Plant Health Technologies), indicating their potential compatibility with this commercial biological control product.

Phage Eram46, mentioned above, may have exhibited a broader host range due to modification activity by *E. amylovora* strain Ea6-4, but this cannot be confirmed. Phage Eram7, a relative of PEa1, exhibited a host range broader than those of other members of group 3. The reason for this is unknown, although Eram7 is the only phage of this type isolated from site 1. Other group 3 phages isolated from different sites exhibit differences in restriction patterns; it is possible that the broadened host range of Eram7 is another variation attributable to geographic separation of isolation sites.

4.2 Biological control with bacteriophages

In the immature pear fruit bioassay, bacteriophages were able to inhibit the ability of *E. amylovora* to produce bacterial exudate, or ooze, on plugs of immature pear fruit (Fig. 12). Phages of groups 3 and 6 exhibited the greatest overall ability to suppress ooze formation, although phages Eram2 and Eram26 from groups 1 and 4, respectively, also expressed a variable ability to suppress ooze. Variations within phage groups may be attributable to differences caused by the pathogen strain used to propagate and test the phages.

Apparent lack of ooze on the pear plug surface did not necessarily imply the absence of bacteria. As can be seen by comparing Figs. 12 and 13, rating plugs of immature pear fruit on the basis of presence or absence of ooze does not directly predict the ability of phage to control bacterial populations on the pear plug surface. Phages Eram2 and Eram26 reduced the incidence of ooze production on the pear plug surface by 50%, as opposed to reductions of 100% and 95% by phages Eram24 and

Eram45, respectively (Fig. 12). It was hypothesized that corresponding differences in these phages' abilities to control the bacterial population on the plug surface would be exhibited. The same four phages, however, reduced the population of *E. amylovora* cells on the plug surface with roughly equal efficacy when the MOI was 1 (Fig. 13).

While reductions in bacterial populations were significant, the population surviving phage treatment was large, numbering from 6×10^7 to 2×10^9 CFU. The experiment was repeated with an increase of the phage concentration to an MOI of 10 (Fig. 14). Significant control of the *E. amylovora* population on the pear plug surface was obtained, but the surviving bacterial population was still high. Additionally, streptomycin, the recommended chemical control agent against *E. amylovora*, failed to produce significant control of the bacterial population on the plug surface.

Physical escape of the pathogen from the phage is the most likely explanation of these results. A dose-response model for biological control systems proposed by Johnson (1994) suggests that the amount of relative disease reduction decreases at greater doses of biological control agent as a proportion of the pathogen remains inaccessible to the activity of the agent. Reductions of the bacterial population to roughly 1×10^8 CFU on the pear plug surface would appear to be the lower limit attainable in this system.

The fact that phage-sensitive bacteria were easily isolated from phage inoculated plugs at the time of evaluation would indicate that mutation to phage resistance was an unlikely means of pathogen inaccessibility. Diffusion of the phage away from the plug surface is a possibility which is supported by the inability of streptomycin to control the *E. amylovora* population on the pear plug surface, an indicator that the control agent may diffuse away rapidly. The degradation of the phage after application to the plug surface may also have contributed to the ability of the bacteria to escape the activity of

the phage. While conditions of moisture and pH on the plug surface were within limits tolerated by most phages (Ackermann and Dubow, 1987a), the production of proteolytic compounds by the ripening fruit may contribute to phage inactivation.

The mechanism by which *E. amylovora* may exist in fairly high numbers on the plug surface and yet produce no visible exudate is not known. The simplest explanation is that a large decrease in the bacterial population on the plug surface corresponds to an equivalent reduction in its volume, and therefore its visibility to the unaided eye. It is possible that exudate was not detected due to the degradation of the bacterial capsule, which makes up a large portion of the volume of ooze (Hartung et al., 1988). In the case of phage PEa1, a soluble capsular depolymerase enzyme is produced in excess during the phage's lytic cycle (Ritchie, 1978; Hartung et al., 1988). Other phages in the collection, such as those in groups 2 and 4, produce a halo surrounding their plaques, which is an indicator of the production of a capsular depolymerase. As a group, however, these phages exhibited reduced abilities to suppress ooze production in the immature pear bioassay. This would indicate that capsular degrading ability is not a major determining factor in preventing the production of ooze.

Evaluation of biological control activity should be repeated using the blossom bioassay system, as this system is a much more accurate model of the environment in which the phage would be expected to exercise biological control of *E. amylovora*. It is for this reason that the results of this bioassay system would be more applicable to field use (Pusey 1997; Schisler and Slininger, 1997). The plug surface may not closely mimic the blossom surface, which undergoes periods of moisture and dessication as well as temperature fluctuations. The plug surface in the experiments conducted here was 26 ºC and exposed to constant moisture.

The stabilization of the phage at the sites inhabited by *E. amylovora* would be highly desirable as this would eliminate one means by which the pathogen may escape the activity of the biological control agent. The ability of some phage isolates to infect *P. agglomerans* strains holds some promise, as this epiphytic bacterium is able to maintain populations in sites which are commonly inhabited by *E. amylovora* (Hattingh et al., 1986). Application of mixtures of phage and selected *P. agglomerans* strains may provide a reservoir of alternate host bacteria which would maintain the phage population in the absence of *E. amylovora*.

4.3 Conclusions

This work represents the initial steps in the production of a bacteriophage-based biological control agent against *E. amylovora*. The molecular characterization of the 45 phage isolates in the collection will aid future work immensely, allowing more rapid classification of phages as they are isolated and permitting further work on the ecology and distribution of the phages of *E. amylovora*. A number of phages related to phage PEa1 were isolated, with some divergence found among the molecular markers of phages isolated from different locations. Some phage types exhibit host ranges limited to certain *E. amylovora* strains, suggesting that care must be taken when evaluating the biological control activity of these phages and when selecting potential bacterial hosts for future phage isolations. The finding that some of the phages of *E. amylovora* are also able to infect strains of *P. agglomerans* will aid immensely in designing future biological control systems which may integrate *P. agglomerans* with phage on the blossom surface.

In the immature pear plug bioassay, phage isolates exhibited significant biological control activity against *E. amylovora*, although the applicability of this data to

field conditions remains to be tested. It is very encouraging that the phages in an unoptimized system are able to reduce bacterial populations on the pear plug surface by almost 100-fold. Results indicate that this bioassay system may not reliably model field conditions, as the bioassay appears to allow large amounts of pathogen escape from the phage, a phenomenon which may not occur under field conditions.

References

Ackermann, H-W. 1996. Frequency of morphological phage descriptions in 1995. Archives of Virology 141: 209-218.

Ackermann, H-W 1987. Bacteriophage taxonomy in 1987. Microbiological Sciences 4: 214-218.

Ackermann, H. W., Audurier, A., Berthiaume, L., Jones, L. A., Mayo, J. A., Vidaver, A. K. 1978. Guidelines for bacteriophage characterization. Advances in Virus Research 23: 1-24.

Ackermann, H-W, Dubow, M. S. 1987. Viruses of prokaryotes, vol. 1: General properties of bacteriophages. CRC Press, Boca Raton.

Ackermann, H-W, Dubow, M. S. 1987a. Viruses of prokaryotes, vol. 2: Natural groups of bacteriophages. CRC Press, Boca Raton.

Adams, M. H. 1959. Bacteriophages. Wiley-Interscience, New York.

Ark, P. A. 1953. Use of streptomycin dust to control fire blight. Plant Disease Reporter 37: 404-406.

Alfano, J. R., Collmer, A. 1996. The type III (Hrp) secretion pathway of plant pathogenic bacteriatrafficking harpins, Avr proteins, and death. Journal of Bacteriology 179: 5655-5662.

Bauske, R. J. 1971. Wind dissemination of waterborne *Erwinia amylovora* from Pyrus to Pyracantha and Cotoneaster. Phytopathology 61: 741-742.

Beer, S. V., Norelli, J. L., Wright, P. 1977. Fire blight epidemiology: factors affecting release of *Erwinia amylovora* by cankers. Phytopathology 67: 1119-1125.

Bennett, R. A., Billing, E. 1978. Capsulation and virulence in *Erwinia amylovora*. Annals of Applied Biology 89: 41-45.

Berrie, A. M., Billing, E. 1996. Hawthorns as a source of fire blight inoculum in English pear and apple orchards. Acta Horticulturae 411: 35-40.

Bradley, D. E. 1965. The morphology and physiology of bacteriophages as revealed by the electron microscope. Journal of the Royal Microscopical Society 84: 257-316.

Burge, W. D., Enkiri, N. K. 1978. Virus adsorption by five soils. Journal of Environmental Quality 7: 73-76.

Burrill, T. J. 1880. Anthrax of fruit trees; or the so-called fire-blight of pear, and twig blight of apple trees. Proceedings of the American Association for the Advancement of Science 29: 583-597.

Campbell, A. 1988. Phage evolution and speciation. *in* The Bacteriophages, vol. 1, R. Calendar, ed. Plenum Press, New York

Chiou, C-S., Jones, A. L. 1991. The analysis of plasmid-mediated streptomycin resistance in *Erwinia amylovora*. Phytopathology 81:710-714.

Civerolo, E. L., Keil, H. L. 1969. Inhibition of bacterial spot of peach foliage by *Xanthomonas pruni* bacteriophage. Phytopathology 59: 1966-1967.

Covey, R. P., Fischer, W. R. 1988. The significance of secondary bloom to fire blight development on Bartlett pears in eastern Washington. Plant Disease 72: 911.

Crosse, J. E., Goodman, R. N., Shaffer, W. H.. 1972. Leaf damage as a predisposing factor in the infection of apple shoots by *Erwinia amylovora*. Phytopathology 62: 176-182.

Crosse, J. E., Hingorani, M. K. 1958. A method for isolating *Pseudomonas mors-prunorum* phages from soil. Nature 181: 60-61.

Dueck, J., Quamme, H. A. 1973. Fire blight in southern Ontario in 1972. Canadian Plant Disease Survey 53: 101-104.

Dulbecco, R., Ginsberg, H. S. 1988. Virology, 2nd ed. J. B. Lippincott Co., Philadelphia.

Erskine, J. M. 1973. Characteristics of *Erwinia amylovora* bacteriophage and its possible role in the epidemiology of fire blight. Canadian Journal of Microbiology 19: 837-845.

Erskine, J. M., Lopatecki, L. E. 1975. *In vitro* and *in vivo* interactions between *Erwinia amylovora* and related saprophytic bacteria. Canadian Journal of Microbiology 21: 35-41.

Gaudriault, S., Brisset, M. N., Barny, M. A. 1998. HrpW of Erwinia amylovora, a new Hrp-secreted protein. FEBS Letters 428: 224-228.

Ge, Q., van der Zwet, T. 1995. Persistence and recovery of endophytic *Erwinia amylovora* in apparently healthy apple tissues. Acta Horticulturae 411: 29-31.

Gill, J. J., Svircev, A. M., Myers, A. L., Castle, A. J. 1999. Biocontrol of *Erwinia amylovora* using bacteriophage. Phytopathology 89: S27. (Abstract)

Harbaugh, B. K., Jones, J. B., Flaherty, J. E., Jackson, L. E. 1998. Beneficial viruses: new allies in the fight against bacterial diseases. GrowerTalks, Oct. 1998: 84-87.

Harrsion, A.., Gibbins, L. N. 1975. The isolation and characterization of a temparate phage, Y46/(E2), from *Erwinia herbicola* Y46. Canadian Journal of Microbiology 21: 937-944.

Hartmann, H. T., Kester. D. E. 1983. Plant Propagation: Principles and Practices, 4th ed. Prentice-Hall Inc., Englewood Cliffs, NJ. 727 pp.

Hattingh, M. J., Beer, S. V., Lawson, E. W. 1986. Scanning electron microscopy of apple blossoms colonized by *Erwinia amylovora* and *E. Herbicola*. Phytopathology 76: 900-904.

He, S. H. 1998. Type III protein secretion systems in plant and animal pathogenic bacteria. Annual Review of Phytopathology, 36: 363-392.

Heuberger, J. W., Poulos, P. L. 1953. Control of fire blight and frog-eye leaf spot (black rot) diseases of apples in Delaware in 1952. Plant Disease Reporter 37: 81-83.

Hickey, K. D., Orolaza-Halbrendt, N., van der Zwet, T. 1999. The presence of endophytic *Erwinia amylovora* bacteria in symptomless apple tissue on orchard trees. Acta Horticulturae 489: 453-458.

Hildebrand, E. M. 1937. Infectivity of the fire blight organism. Phytopathology 27: 850-852.

Jackson, H. S. 1910. Fire Blight of Pear and Apple. Oregon Agricultural College Press, Curcular Bulletin No. 7.

Janisiewicz, W. J. 1999. Biocontrol of postharvest diseases of temparate fruits. *in* Boland, G. J., Kuykendall, L. D., eds., Plant-Microbe Interactions and Biological Control. Marcel Dekker, Inc., New York.

Jensen, E. C., Schrader, H. S., Rieland, B., Thompson, T. L., Lee, K. W., Nickerson, K. W., Kokjohn, T. A. 1998. Prevalence of broad-host-hange lytic bacteriophages of *Sphaerotilus natans, Escherichia coli*, and *Pseudomonas aeruginosa*. Applied and Environmental Biology 64: 575-580.

Johnson, K. B. 1994. Dose-response relationships and inundative biological control. Phytopathology 84:

780-784.

Johnson, K. B., Stockwell, V. O. 1998. Management of fire blight: A case study in microbial ecology. Annual Review of Phytopathology 36: 227-248.

Jones, A. L. 1992. Evaluation of the computer model MARYBLYT for predicting fire blight blossom infection on apple in Michigan. Plant Disease 76: 344-347.

Jones, J. B., Somodi, G. C., Jackson, L. E., Harbaugh, B. K. 1998. Control of bacterial spot on tomato in the greehouse and field with bacteriophages. 7th International Congress of Plant Pathology, Edinburgh, Scotland, August 1998: Paper 5.2.14.

Kearns, L. P., Hale, C. N. 1996. Partial characterization of an inhibitory strain of *Erwinia herbicola* with potential as a biocontrol agent for *Erwinia amylovora*, the fire blight pathogen. Journal of Applied Bacteriology 81: 369-374.

Kim, J. F., Wei, Z. M., Beer, S. V. 1997. The hrpA and hrpC operons of *Erwinia amylovora* encode components of a type III pathway that secretes harpin. Journal of Bacteriology 179: 1690-1697.

Lindow, S. 1998. Staggered sprays of biocontrol agent best. California Grower, Jan. 1998: 10-11.

Loper, J. E., Henkels, D., Roberts, R. G., Grove, G. G., Willet, M. J., Smith, T. J. 1991. Evaluation of streptomycin, oxytetracycline and copper resistance of *Erwinia amylovora* isolated from pear orchards in Washington state. Plant Disease 75: 287-290.

Mandell, J. D., Eisenstark, A. 1953. Factors affecting plaque formation by *Xanthomonas pruni* bacteriophage. Phytopathology 43: 27-29.

Manfioletti, G., Schneider, C. 1988. A new and fast method for preparing high quality lambda DNA suitable for sequencing. Nucleic Acids Research 16: 2873-2884.

Maniloff, J., Ackermann, H-W. 1998. Taxonomy of bacterial viruses: establishment of tailed virus genera and the order *Caudovirales*. Archives of Virology 143: 2051-2063.

McManus, P. S., Jones, A. L. 1994. Role of wind driven rain, aerosols, and contaminated budwood in incidence and spatial pattern of fire blight in an apple nursery. Plant Disease 78:1059-1066.

McManus, P. S., Jones, A. L. 1994a. Epidemiology and and genetic analysis of streptomycin-resistant *Erwinia amylovora* from Michigan and evaluation of oxytetracycline for control. Phytopathology 84:627-633.

Miller, T. D., Schroth, M. N. 1972. Monitoring the epiphytic population of *Erwinia amylovora* on pear with a selective medium. Phytopathology 62: 1175-1182.

Moller, W. J., Beutel, J. A., Reil, W. O., Zoller, B. G. 1972. Fire blight resistance to streptomycin in California. Phytopathology 62: 779 (Abstract).

Momol, M. T., Norelli, J. L., Aldwinckle, H. S., Breth, D. I. 1999. Internal movement of *Erwinia amylovora* from infection in the scion and economic loss estimates due to the rootstock phase of fire blight of apple. Acta Horticulturae 489: 505-507.

Momol, M. T., Norelli, J. L., Piccioni, D. E., Momol, E. A., Gustafson, H. L., Cummins, J. N., Aldwinckle, H. S. 1998. Internal movement of Erwinia amylovora through symptomless apple scion into the rootstock. Plant Disease 82: 646-650.

Munsch, P., Olivier, J. M. 1995. Biocontrol of bacterial blotch of the cultivated mushroom with lytic

phages: Some practical considerations. *in* Elliott, T. J., ed, Science and Cultivation of Edible Fungi, Vol. II: Proceedings of the 14th International Congress, Oxford, 17-22 September 1995: 595-602.

Nelson, M., McClelland, M. 1991 Site-specific methylation: effect on DNA modification methyltranseferases and restriction endonucleases. Nucleic Acids Research 19: 2045-2071.

Okabe, N., Goto, M. 1963. Bacteriophages of plant pathogens. Annual Review of Phytopathology 1: 397-418.

OMAFRA, 1996. 1996/1997 Fruit Production Recommendations. Ontario Ministry of Agricultire, Food and Rural Affairs Publication 360.

Orton, C. R., Adams, J. F. 1915. Collar Blight and Related Forms of Fire Blight. Pennsylvania State College School of Agriculture, Bulletin No. 136.

Palmer, E. L., Fernando, W. G. D., Jones, A. L. 1997. Control of *Erwinia amylovora* by mixtures of bacteriophage. Phytopathology 87: S73-S74 (Abstract).

Pelkonen, S., Aalto, J., Finne, J. 1992. Differential activities of bacteriophage depolymerase on bacterial polysaccharide: Binding is essential but degradation is inhibitory in phage infection of K1-defective *Escherichia coli*. Journal of Bacteriology 174: 7757-7761.

Pusey, P.L. 1999. Effect of nectar on microbial antagonists evaluated for use in control of fire blight of pome fruits. Phytopathology 89: 39-46.

Pusey, P. L. 1997. Crab apple blossoms as a model for research on biological control of fire blight. Phytopathology 87: 1096-1102.

Reil, W. O., Moller, W. J., Thomson, S. V. 1979. An historical analysis of fire blight epidemics in the central valley of California. Plant Disease Reporter 63: 545-549.

Ritchie, D. F., Klos, E. J. 1979. Some properties of *Erwinia amylovora* bacteriophages. Phytopathology 69: 1078-1083.

Ritchie, D. F., Klos, E. J. 1976. Isolation of *Erwinia amylovora* bacteriophage from aerial parts of apple trees. Phytopathology 67: 101-104.

Ritchie, D. F. 1978. Bacteriophages of *Erwinia amylovora*: Their Isolation, Distribution, Characterization, and Possible Involvement in the Etiology and Epidemiology of Fire Blight. PhD dissertation, Department of Botany and Plant Pathology, Michigan State University.

Schisler, D. A., Slininger, P. J. 1997. Microbial selection strategies that enhance the likelihood of developing commercial biological control products. Journal of Industrial Microbiology and Biotechnology, 19: 172-179.

Schnabel, E. L., Fernando, W. G. D., Jackson, L. E. 1999. Bacteriophage of *Erwinia amylovora* and their potential for biocontrol. Acta Horticulturae 489: 649-653.

Schroth, M. N., Thomson, S. V., Moller, W. J. 1978. Streptomycin resistance in *Erwinia amylovora*. Phytopathology 69: 565-568.

Spangelo, L. P. S., Watkins, R., Davies, E. J. 1968. Fruit Tree Propagation. Canada Department of Agriculture, Publication 1289.

Stewart, V. B. 1913. Fire Blight Disease in Nursery Stock. Cornell University Agricultural Experiment

Station, Bulletin 329.

Steiner, P. W. 1998. Fire blight symposium: Copper treatments. Pennsylvanis Fruit News, 78: 86-87.

Steiner, P. W., Lightner, G. W. 1992. MARYBLYT: A predictive program for forecasting fire blight disease in apples and pears. Version 4.0. University of Maryland, College Park.

Stockwell, V. O., Johnson, K. B., Loper, J. E. 1998. Establishment of bacterial antagonists of *Erwinia amylovora* on pear and apple blossoms as influenced by inoculum preparation. Phytopathology 88: 506-513.

Thomas, H. E., Ark, P. A. 1934. Nectar and rain in relation to fire blight. Phytopathology 24: 682-685.

Thomson, S. V. 1992. Fire blight of apple and pear. *in* Kumar, J., Chaube, H. S., Singh, U. S., Mukhopadhyay, eds, Plant Diseases of International Importance, vol. III: Diseases of Fruit Crops. Prentice hall, Englewood Cliffs.

Thomson, S. V. 1986. The role of the stigma in fire blight infections. Phytopathology 76: 476-82.

Thomson, S. V., Hansen, D. R., Flint, K. M., Vandenberg, J. D. 1992. Dissemination of bacteria antagonistic to *Erwinia amylovora* by honey bees. Plant Disease 76: 1052-1056.

Thomson, S. V., Schroth, M. N., Moller, W. J., Reil, W. O. 1982. A forecasting model for fire blight of pear. Plant Disease 66: 576-579.

Thomson, S. V., Schroth, M. N., Moller, W. J., Reil, W. O. 1976. Efficacy of bactericides and saprophytic bacteria in reducing colonization and infection of pear flowers by *Erwinia amylovora*. Phytopathology 66: 1457-1459.

Thomson, S. V., Wagner, A. C., Gouk, S. C. 1999. Rapid epiphytic colonization of apple flowers and the role of insects and rain. Acta Horticulturae 489: 459-464.

Travis, J. W., Kleiner, W. 1997. Evaluation of techniques for removal of fire blighted shoots. Pennsylvania Fruit News 77: 48-50.

Travis, J. W., Rytter, J. L., Hickey, K. D. 1998. The susceptibility of various apple rootstocks to *Erwinia amylovora*, the causal agent of fire blight. Pennsylvania Fruit News 78: 43-45.

van der Zwet, T. 1996. Present worldwide distribution of fire blight. Acta Horticulturae 411: 7-8.

van der Zwet, T., Beer, S. V. 1995. Fire Blight—Its Nature, Prevention and Control. United States Department of Agriculture, Agriculture Information Bulletin 631.

van der Zwet, T., Biggs, A. R., Heflebower, R., Lightner, G. W. 1994. Evaluation of the MARYBLYT computer model for predicting blossom blight on apple in West Virginia and Maryland. Plant Disease 78: 225-230.

van der Zwet, T., Keil, H. L. 1979. Fire Blight: A Disease of Rosaceous Plants. United States Department of Agriculture, Agriculture Handbook 510.

Vanneste J. L., Harper G. E., Perry J. H., Ede F. J., Popay A. J. 1995. A bioassay to measure in the laboratory the inhibition of fire blight by different biological control agents and their derivatives. Proceedings of the Forty Eighth New Zealand Plant Protection Conference, Angus Inn, Hastings, New Zealand, August 8-10, 1995: 158-160.

Vanneste J.L., Yu J., and Beer S.V. 1992. Role of antibiotic production by *Erwinia herbicola* Eh252 in biological control of *Erwinia amylovora*. Journal of Bacteriology 174: 2785-2796.

Vanneste J. L., Yu J., Harper G. E., Perry J. H., Bonn W. G. 1996. Plugs of immature pear fruit to assess the virulence of *Erwinia amylovora* and to study in the laboratory the interaction between biological control agents and the fire blight pathogen. Acta Horticulturae 411: 303-307.

Van Regenmortel, M. H. V., Bishop, D. H. L., Fauquet, C. M., Mayo, M. A., Maniloff, J., Calisher, C. H. 1997. Guidelines to the demarcation of virus species. Archives of Virology, 142: 1505-1518.

Vidaver, A. K. 1976. Prospects for control of phytopathogenic bacteria by bacteriophages and bacteriocins. Annual Review of Phytopathology 13: 451-465.

Williams, S. T., Mortimer, A. M., Manchester, L. 1987. Ecology of soil bacteriophages. *in* Goyal, S. M., Gerba, C. P., Bitton, G., eds, Phage Ecology, Wiley-Interscience, New York.

Wilson, M., Epton, H. A. S., Sigee, D. C. 1992. Interactions between *Erwinia herbicola* and *E. amylovora* on the stigma of hawthorn blossoms. Phytopathology 82: 914-918.

Wilson, M., Lindow, S.E. 1993. Interactions between the biological control agent *Pseudomonas fluorescens* A506 and *Erwinia amylovora* in pear. Acta Horticulturae 338: 329-330.

Zeller, W., Wolf, B. 1996. Studies on biological control of fire blight. Acta Horticulturae 411: 341-345.

Zoller, B. G., Sisevich, J. 1979. Blossom popultions of *Erwinia amylovora* in pear orchards versus accumulated degree hours over 18.3 °C (65 F), 1972-1976. Phytopathology 69: 1050 (Abstract).